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Digestive Action of Human Gastric Juice.* (22439)

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Perfusion experiments carried out by a number of workers(1-3) have shown the digesting and ulcerating effects of hydrochloric acid-pepsin solutions on living tissues, and the importance of pepsin in the production of such digestion has been emphasized. Dameron and Wangensteen(4) demonstrated the greater necrotizing effect in intestinal loops of canine gastric juice obtained from vagally innervated pouches under pilocarpine stimulation than that from Heidenhain pouches stimulated by histamine. The more severe degrees of damage produced by the former were assumed to be due to a greater peptic activity. However, we have found no reference to the study of the peptic activity of human gastric juice by perfusion experiments of this type.

The present investigation was carried out to determine the digestive action *in vivo* of human gastric juice with simultaneous measurement of peptic activity and acidity. The results indicate that the gastric secretions of patients with duodenal ulcer, patients who are acutely ill, and patients in the early postoperative period secrete gastric juice with a greater digestion power than that of control patients and that this increased digestive action is due to increased peptic activity of the juice.

Method. Gastric secretion was collected from fasting patients by an inlying gastric tube

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TABLE I. 8 Hour Night Secretion of Gastric Juice of Patients in Different Categories.

Category	Total No. of patients	Volume (cc) Mean	Volume (cc) Range	Free HCl (total Meq.) Mean	Free HCl (total Meq.) Range	Peptic activity — Tyrosine units/cc Mean	Tyrosine units/cc Range	Pepsin,* mg/cc Mean	Pepsin,* mg/cc Range	pH Mean	pH Range	% patients with pH 2.0 or below
Control	53	263	10– 860	2.87	0–19.05	1.0	.1 –5.0	.55	.06–1.85	3.9	1.6–6.8	16%
Duodenal ulcer	18	467	48– 960	20.90	0–63.65	1.7	.15–4.1	.89	.08–1.68	2.3	1.6–4.2	53
Gastric "	9	384	90– 750	21.89	0–60.0	1.4	.24–3.0	.75	.14–1.4	2.7	1.4–6.7	50
Acutely ill	9	549	240–1310	15.50	0–78.6	1.6	.55–2.4	.85	.29–1.19	2.4	1.0–4.0	63
Postoperative	64	313	40–1100	7.60		1.4	.1 –5.2	.75	.06–1.87	3.8	1.5–7.7	29
Gastric cancer	16	224	5– 850	.84	0–13.0	.6	.1 –1.8	.32	.06– .95	5.4	1.7–7.6	6.7

* Expressed as mg/cc, 3 × crystallized bovine pepsin.

attached to a water siphon. The collecting bottle was kept on ice during the 8-hour period of aspiration from 11 p. m. to 7 a. m. The volume was measured and a sample removed for pH, acid, and pepsin determination. The pH was measured by the Northrup and Leeds glass electrode, pepsin was determined by the hemoglobin substrate method of Anson(5); free acid was measured by titration with .1 N NAOH. The pH of the remainder was adjusted to 1.6-1.7 with hydrochloric acid to provide an optimal medium for peptic activity. It was then used to perfuse the esophagus of the living anesthetized cat. The extensive susceptibility of the cat esophagus to the digestive action of gastric juice was demonstrated by Ferguson and associates(6). The sample was used for perfusion immediately or preserved below 0°C until used. Cats were anesthetized with pentobarbital (30 mg per kg) and a cannula was tied into the cervical esophagus and another into the gastric cardia. Perfusion of the cat's esophagus was carried out by dripping the gastric juice from a reservoir bottle at a rate of 25-35 ml per hour. The outflow tubing was raised to 20 cm above the level of the esophagus to provide a constant intraesophageal pressure. At the end of a 2-hour period the animal was sacrificed and the esophagus carefully examined. The degree of necrosis of the esophagus was graded as follows on the basis of gross and microscopic examinations: Grade 0: No injury or only hyperemia of the esophageal mucosa; Grade 1: Erosion of mucosal epithelium; Grade 2: Ulceration into submucosa; Grade 3: Digestion into muscular layers of esophagus; Grade 4: Impending perforation; Grade 5: Perforation during the perfusion period. Patients whose gastric secretions were studied were divided into the following groups: a) *Controls*: Patients admitted to hospital who were not acutely ill and who did not have duodenal ulcer, gastric ulcer or other gastric disease. b) *Duodenal ulcer*: Patients hospitalized on the Medical or Surgical Service for treatment of duodenal ulcer. All had clinical and radiographic evidence of active duodenal ulcer. c) *Gastric ulcer*: Patients hospitalized for treatment of

TABLE II. Results of Perfusion of Cat Esophagus with Human Gastric Juice.

Category	Total No. of perfusions	Grade of digestion 0	1	2	3	4	5
Control	32	5	19	5	1	1	1
Duodenal ulcer	18	1	3	1	3	5	5
Gastric "	9	1	5	2	—	—	1
Postoperatives	54	4	22	14	3	1	10
Acutely ill	7	—	1	1	4	—	1
Gastric cancer	9	2	5	2	—	—	—
HCl in distilled water, pH 1.6	5	4	1	—	—	—	—

a gastric ulcer believed to be benign. All except one of the group subsequently were operated upon and were shown to have a benign gastric ulcer. The remaining patient refused operation. d) *Gastric cancer*: Patients later demonstrated to have gastric carcinomas by operation. e) *Acutely ill*: Patients who, by clinical criteria, were known to be acutely ill at the time of gastric aspiration, but had not been operated upon recently, and did not have duodenal ulcer or a gastric lesion. (f) *Postoperative*: Patients who had undergone major extragastric surgical procedures one to 5 days prior to the period of gastric aspiration.

Results. Table I shows the results obtained by analysis of 8-hour night secretions for volume, free HCl, pepsin, and pH. Mean volumes were increased above control levels to a significant degree only in duodenal ulcer and acutely ill patients.

Peptic activity, expressed as tyrosine units (5), was significantly increased in the gastric secretions of patients with duodenal ulcer, and patients in the postoperative period. The mean value obtained for the acutely ill group is increased above control level, but is not significant ($t = 1.683$ $p = .1$). There was no significant difference in peptic activity in gastric juice of gastric ulcer and gastric cancer patients as compared with control values.

Mean pH values were significantly lower than the control value in the duodenal ulcer, gastric ulcer, and acutely ill patients. In addition, a greater percentage in each of these groups and the postoperative group had pH values optimal for peptic activity (pH 2 or below).

Table II shows the results obtained by perfusion of the cat esophagus with the gastric juice obtained from patients in various categories. Each perfusion was carried out with gastric juice of a different patient. Fig. 1 illustrates minimal (Grade 1) and severe (Grade 5) esophageal digestion produced by 2 hour perfusion. Gastric secretion from the majority of control patients produced only lesser degrees of esophageal damage. However, secretions obtained from 72% of patients with duodenal ulcer produced Grade 3 to 5 esophageal digestion. Likewise, the gastric secretions from patients in the early postoperative period caused greater esophageal digestion than normal. In this group 26% of specimens caused moderate to severe esophageal damage. Gastric aspirate from acutely ill patients shows a similar increased digestive power of the gastric juice. The differences in digestive action of gastric juice of patients in these three categories are significant as compared to that of controls. Except for one patient, gastric juice from persons with gastric ulcer caused no greater digestion of the cat's esophagus than that of control patients. Additional observations will be made upon patients with gastric ulcer as they become available for study. Gastric secretions obtained from persons with gastric cancer showed no variation in necrotizing effects from that of control patients.

Discussion. The finding of an increased digestion action on living tissue by the gastric secretions of duodenal ulcer patients is in agreement with data obtained from *in vitro* studies which have shown increased peptic activity in the gastric juice(7-9). However, although the number of patients available thus far has been small, results obtained suggest that gastric ulcer is not associated with increased digestive power of the gastric secretions. This would favor the concept that the pathogenesis of gastric ulcer and that of duodenal ulcer differ from each other.

The increased necrotizing action caused by gastric juice of acutely ill patients and patients in the early postoperative period supports the idea that ulcers of the upper gastrointestinal tract occurring during such stress periods are the result of increased proteolytic

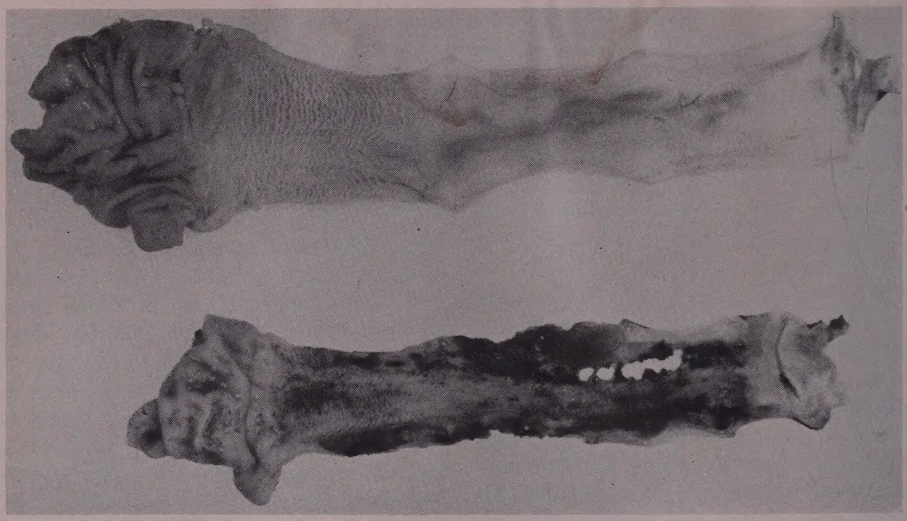

FIG. 1. (Upper): Grade 1 digestion of cat esophagus. Gastric juice from control patient. (Lower): Severe digestion and perforation of esophagus (Grade 5). Gastric juice from duodenal ulcer patient.

activity of the gastric juice rather than a decreased resistance of the mucosa of the intestine to ulceration. The results are in accord with the observations of Gray(10) and others that stress is accompanied by increased peptic activity of the gastric secretions. In addition, an optimal acid medium for peptic activity(2) is present in a greater proportion of the gastric secretions obtained from patients of these 2 groups.

Summary. 1. The digestive capacity of human gastric juice, adjusted to pH 1.6-1.7, has been measured by perfusion of the cat's esophagus. 2. The gastric secretions of patients with duodenal ulcer, patients in the early postoperative period, and acutely ill patients showed significantly increased necrotizing effects on the esophagus as compared with controls. Gastric secretions from patients with gastric cancer and benign gastric ulcer showed no such increased digestive activity. 3. Peptic activity measured *in vitro* was significantly increased in duodenal ulcer patients and patients in the postoperative period. The mean pH values were significantly lower than control values in duodenal and gastric ulcer patients, postoperative patients and acutely ill patients.

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Method for Determining Iron-Binding Capacity of Serum.* (22440)

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(Introduced by Edwin L. Smith.)

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The availability of radioactive iron has permitted laboratory evaluation of hematopoietic disorders based upon the rate and quantity of iron transport by the serum, and the subsequent distribution of this iron(1,2). To interpret the data obtained from iron turnover studies, it must be established that the capacity of the serum to bind iron is not a limiting factor by demonstrating residual iron-binding capacity. The serum iron concentration and residual binding capacity of normal persons and of patients with a variety of diseases have been determined using several published chemical and isotopic procedures(3-5). The simplicity of the method described here for analyzing for serum iron-binding capacity has permitted this laboratory to obtain reproducible values on a large number of samples. Iron labelled with a radioactive tracer is added to a serum sample in excess of the binding capacity, and that which becomes attached is separated by protein precipitation and measured isotopically. Preliminary studies indicated several difficulties. Ionic iron will not remain in solution at neutral pH, but precipitates as ferric hydroxide, which has a solubility product ($k_s.p.$), of 1.1×10^{-36} (18°C). Several authors have advocated addition of iron chloride in acid solution directly to serum to obtain protein-bound iron, since the buffering capacity of serum is adequate to neutralize small volumes of weakly acid solutions. If this is done, the iron is not dialysable, and it precipitates with the protein fraction. Nevertheless, much of the added iron is in the hydroxide form, and will not permit determination of iron-binding capacity, nor behave as a tracer for protein-bound iron *in vivo*. Iron complexes which are stable at neutral pH may be used, but the complex must not be so stable as to inhibit transfer of iron to the iron-binding protein fraction of serum (IV-7, Cohn). A preliminary study indicated that iron will be distributed between equal quantities, by weight, of citrate and of iron-binding protein in a ratio of one to seventy. Gluconate binds iron somewhat more firmly than does citrate, and versenate has even greater affinity for iron than does the protein. The most satisfactory agent was found to be ascorbic acid in small concentration. To separate the serum proteins, ammonium sulfate is the precipitating agent of choice(4). The colloidal suspension is easily precipitated by ethanol, facilitating separation by centrifugation. Washing the precipitate is not necessary, since isotope studies have shown that less than 0.1 ml of the supernatant containing less than 4% of the excess iron is occluded. Filtration is laborious and time consuming, and often subject to errors of adsorption. Heparinized plasma cannot be used in place of serum, as there is apparently an interference with iron transfer causing false low iron-binding capacity values to be obtained.

Method. A. *Reagents*: (1) Carrier-radioiron chloride solution: $Fe^{59}Cl_3$ in HCl is diluted to approximately 0.2 μc/ml. Reagent grade $FeCl_3 \cdot 6H_2O$ is dissolved in the solution to give a total iron concentration of about 60 μg/ml, and standardized against a solution made from iron wire dissolved in HCl. This solution may be stored in polyethylene bottles. If iron dissolved in HCl is used instead of $FeCl_3$ crystals, the total acidity of the final preparation (solution #2) must not exceed the buffering capacity of the serum. (2) Carrier-radioiron ascorbate solution: About 10 mg of pure crystalline ascorbic acid is dissolved in 1.0 ml of solution #1 just prior to use. (3) Saturated ammonium sulfate solution: 530 g of reagent grade $(NH_4)_2SO_4$ is dissolved in slightly less than

* This work was aided in part by Medical Research and Development Board, Office of Surgeon General, Department of Army.

one liter of iron-free water. If pH is less than 6 it is adjusted to 6.0 with saturated NaOH solution, and diluted to a final volume of one liter. The pH of this solution must be checked occasionally since it may drop due to loss of NH_3 on long standing. (4) 95% ethanol. B. *Procedure*: New glassware is maintained free from iron by washing with a detergent, rinsing once thoroughly in tap water and finally twice with distilled water. *Technic.* 1. Measure 0.1 ml radioiron ascorbate solution into a pyrex test tube 15 x 125 mm. 2. Add 1 ml serum and mix well. Allow to stand for 10 minutes at room temperature and determine the radioactivity. 3. Add 10 ml of $(NH_4)_2SO_4$ solution, mix by shaking, and allow to stand for one hour. 4. Add 1 ml ethanol, invert several times and centrifuge for 10 minutes at about 1400 x g. 5. With a fine glass stirring rod, gently tilt the protein plug and while holding it against the wall of the tube, carefully pour off supernatant. Push the plug to the bottom of the tube and loosen any precipitate adhering to the walls of the tube with the stirring rod, then rinse down the rod and walls of the tube with 1 ml distilled water. 6. Count the precipitate and calculate the iron-binding capacity according to the formula: $\frac{\text{Counts of precipitate}}{\text{Original counts added}} \times \mu g$ Fe added x 100 = μg% binding capacity.

The method was evaluated as follows: 1. Tagged iron was added to one ml samples of pooled patient sera in successive increments of 0.5 μg, allowed to incubate 10 minutes after which 6 μg of nonradioactive iron as ascorbate was added, and the radioiron in the precipitated protein of each was measured. Results of this experiment show that the radioiron added to the unsaturated protein remains attached during the test. Thus, on adding 0.50, 1.00, 1.50, 2.00, 2.50 and 3.00 μg of Fe ascorbate to the serum, 0.47, 0.93, 1.40, 2.03, 2.30 and 2.37 μg were found in the protein plug. In the last 2 experiments the serum was saturated with iron. 2. Pooled patient sera samples were saturated with nonradioactive iron ascorbate, and radioiron ascorbate added thereafter to prove that appreciable exchange does not take place between bound iron and unbound iron during the procedure. Upon adding 0.50, 2.50 and 5.00 μg of radioactive Fe ascorbate to the serum, 0.04, 0.18 and 0.26 μg of radioactive Fe were measured in the protein plug. 3. Graded increments of nonradioactive iron ascorbate were added to pooled sera samples, and residual iron-binding capacities measured. In this series, in which the original iron-binding capacity was 296 μg %, the addition of 50, 100, 150, 200, 250 and 500 μg % of cold Fe ascorbate left residual binding capacities which were less than the original value by 46, 103, 153, 206, 246 and 274 μg % respectively.

Summary. 1. A simple radioisotopic method is described for determining the iron-binding capacity of serum. 2. In principle, iron labelled with a radioactive tracer is added to a serum sample in excess of the binding capacity, and that which becomes attached is separated by protein precipitation and measured isotopically. 3. The method was evaluated by measurement of: (a) the recovery of radioiron added in increments to serum samples, (b) the amount of iron exchanged between the saturated iron proteinate and the excess iron in solution, and (c) the difference in residual binding capacities from the original value after the addition of increments of nonradioactive Fe ascorbate to serum samples.

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Effect of Sweating and Changes in Blood Flow on Heating of Human Skin. (22441)

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Much of the experimental work on heat injury concentrates around the nature and quantitation of the stimulus and neglects the nature and quantitation of the tissue response. This report is an attempt to use a controlled intensity of thermal radiation and to examine the effects of sweating and changes in blood flow on the rate of heating of the skin.

Methods. Four cm^2 of the volar side of forearm was exposed to thermal radiations produced by 1,000 watt projection lamp. The intensity was 100 $mc/sec/cm^2$, with wave lengths of 0.5-3.8 μ with peak at 1.5 μ. Due to difficulties of determining reflection and absorption of normal human skin, the evaluation is limited to comparison of various physiological conditions. However, if the skin is painted with India ink, 96% of incident heat energy is absorbed at skin surface. Transmission from India ink into the skin is mainly by conduction. If intensity of incident radiation and temperature rise of skin are known, the thermal characteristics of skin can be calculated. A rapidly recording radiometer measured surface temperature of the skin(1). Heating of a body is determined by conductivity (k), density (ρ), and specific heat (c). The product of these heating characteristics was calculated from the formula: $k\rho c = \frac{1.13\ Q^2t}{\Delta T^2}$, in which 1.13 = constant for blackened human skin; Q = intensity of incident radiation in $mcal./sec./cm^2$; t = time of exposure in sec.; ΔT = difference between skin temp. at time t and initial skin temp. in °C. The formula is based on Buettner's work(2) and indicates that, for a given intensity of radiation absorbed at tissue surface, the greater the $k\rho c$ the less will be the surface temperature rise over a given time interval (3). Heat loss by convection by flowing blood and by vaporization from the skin are not included in the formula. Therefore, the $k\rho c$ concept is used here as an indication of physiological changes. Subcutaneous temperatures were measured with steel-constantan loop thermocouple, ten thousandths of an inch in diameter, soldered end to end and drawn through the skin at a depth of about 1 mm. No attempt was made to measure subcutaneous temperature while skin was exposed to radiation. Subjects for these experiments were laboratory workers between ages 23-42 years. While one arm served as control, the following procedures were carried out on the other arm: 1. Changes of rate of sweating(4) produced by: a. Intradermal inj. of 0.1 cc of atropine (1.25 mg/cc to inhibit sweating; b. Intradermal inj. of 0.1 cc of acetylcholine (100 mg/cc) to increase sweating; c. Intradermal inj. of 0.1 cc of saline as control. The bleb formed by intradermal injection was about 0.5 cm diameter. The effect of the 2 drugs on rate of sweating covered an area considerably larger than 16 cm^2 field of experimental measurement. Changes of blood flow and blood content were produced by: a. Total obstruction of blood flow: arm was raised above heart level for 1 minute and pressure cuff applied above elbow with pressure of 200 mm Hg; b. Venous congestion: pressure in cuff was 80 mm Hg; c. Reactive hyperemia: on release of pressure cuff following procedures a and b, a deep flush of reactive hyperemia which persisted about 3 minutes.

Results. Influence of sweating on heating of skin. Fig. 1 shows the increase of skin temperature with time for a radiation intensity of 100 $mc/sec/cm^2$, and decrease of temperature when the heating was stopped upon reaching the pain threshold. With atropine (inhibition of sweating) rates of increase and of decrease are markedly enhanced. Acetylcholine (increased sweating) produces the opposite result. With acetylcholine, the $k\rho c$ was 82% higher, and with atropine 14%

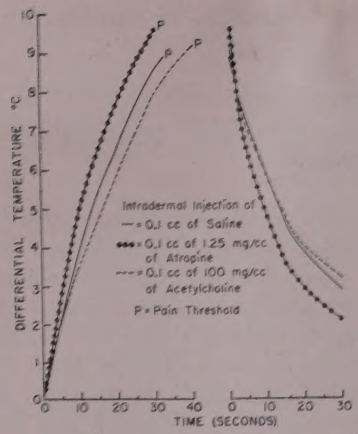

FIG. 1. Influence of pharmacologically induced sweating on heating of normal white human skin.

lower, than the control. The damaging effect of heat is a function of temperature and time of exposure of the skin above 45°C(5,6). Therefore, to determine injurious effects of heat, results are plotted in terms of temperature instead of temperature rise above initial skin temperature. This was done in Fig. 2. The initial skin temperature of the atropine experiment was higher, and that of the acetylcholine experiment lower than the control. This difference of initial skin temperature increases individual differences of heating curves. Even if no actual injury is produced in these experiments, the results are considered an indication that sweating has a protective value against thermal radiation injury.

Influence of changing blood flow and blood supply. Fig. 3 shows effect of total obstruction of blood flow with and without thermal radiation. Epidermal and subcutaneous temperatures during total obstruction of blood

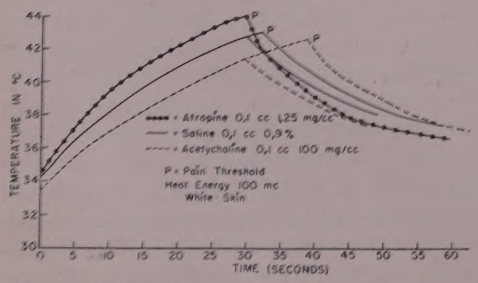

FIG. 2. Influence of sweating on temperature of normal skin exposed to thermal radiation.

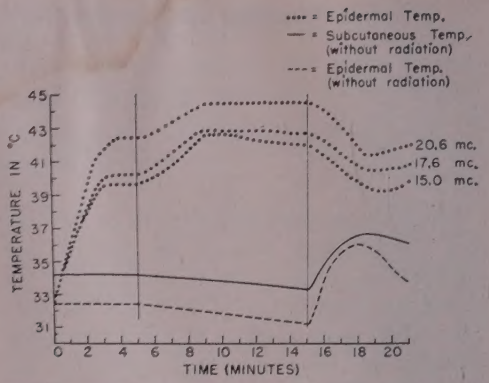

FIG. 3. Effect of prolonged total obstruction of blood flow on normal skin exposed to various levels of thermal radiation.

flow but without radiation are shown at bottom of the graph. Subcutaneous temperature in general follows the epidermal temperature, but to a less extent. Both fall on obstruction and increase on release of pressure cuff. If the arm is exposed to various levels of heat energy, a temperature change in the opposite direction occurs. Before pressure is applied, the temperature goes up and finally comes to an equilibrium. On occlusion, temperature rises again and comes to an equilibrium at a higher level. On release of the pressure cuff, there is a marked decrease in temperature. The $k\rho c$ value goes down during the obstruction and up again during the reactive hyperemia (Table I).

Fig. 4 shows a similar experiment on the effect of venous congestion. The epidermal and subcutaneous temperatures show very lit-

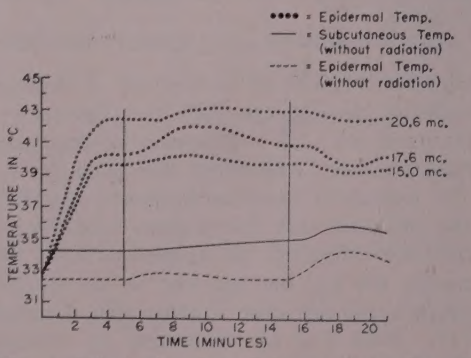

FIG. 4. Effect of prolonged venous obstruction on normal skin exposed to various levels of thermal radiation.

tle change during the experiment and a slight rise during the period of reactive hyperemia. The effects of thermal radiation are not very marked either. The $k\rho c$ in these 2 experiments was determined in 2 different ways: first, the skin (India ink) was exposed to 100 mc/sec/cm^2 of thermal radiation and the increase in skin temperature was determined; second, the skin was exposed to a constant radiation of 15 mc/sec/cm^2 and, intermittently, the energy was increased to 100 mc/sec/cm^2. As far as relative difference between control and various experimental procedures is concerned, the two methods agree fairly well (Table I). However, there is a difference in absolute level of the 2 methods of determination, indicating that heating of the skin increases the $k\rho c$, independent of the other experimental procedures.

TABLE I.* Influence of Blood Flow and Blood Content on Heating of the Skin.

	(1)	(2)	(3)		(4)	(5)	(6)	(7)	(8)
	Epidermal temp.	Subcut. temp.	Equilibrium skin temp. 14 mc/sec/cm^2	17.6 mc/sec/cm^2	20.6 mc/sec/cm^2	Pain threshold	Differential temp. ($T_f - T_o$)	Time to pain threshold ($T_p - T_r$), sec	$k\rho c \times 10^{-5}$ normal skin	$k\rho c \times 10^{-5}$ radiated skin
	°C							cal^2/cm^4/°C^2/sec	
No. of determinations	12	4	4	4	4	8	8	8	8	4
(a) Control	32.4	34.2	39.6	40.2	42.4	44.2	11.8	40	77 ± 2.5	102
(b) Total obstruction	31.2	35.3	42.6	42.8	44.5	44.7	13.5	51	64 ± 2.9	99
(c) Passive hyperemia	34.6	35.9	39.2	40.4	41.3	44.9	10.3	37	85 ± 3.4	112
(d) Venous congestion	32.8	35.0	40.2	42.0	43.1	45.0	12.2	41.6	72 ± 4.7	102
(e) Passive hyperemia	34.2	35.9	39.2	39.6	42.4	44.6	10.4	34.6	78 ± 3.5	109
(f) $\frac{(b)+(d)}{2}$ †	32.0	35.15	41.4	42.4	43.8	44.85	12.85	46.3	68	100.5
(g) (d) – (b) Blood content	+1.6	–.3	–2.4	– .8	–1.4	+.3	–1.3	–9.4	+ 8	+ 3
(h) (e) – (f) Blood flow	+2.6	–.65	–2.2	–2.0	–2.5	+.05	–2.55	–9.3	+17	+11.5

* Each figure represents mean of 8 determinations. † Derived value for normal blood content without blood flow.

FIG. 5. Effect of blood flow on the heating of normal skin.

Fig. 5 gives the rise above initial skin temperature under the 3 experimental conditions, and the return towards normal after 30 seconds of heating. Reactive hyperemia with the highest $k\rho c$ shows the slowest rise of temperature, and total obstruction with the lowest $k\rho c$ shows the highest temperature. However, Fig. 6 indicates that the initial temperature is so high in reactive hyperemia and so low in total obstruction that the final tem-

FIG. 6. Effect of blood flow on temperature of normal skin exposed to thermal radiation.

perature after 30 seconds of exposure is influenced more by the difference in the initial temperature than by difference in the $k\rho c$ values.

Discussion. As $k\rho c$ of water is higher than that of the skin(6), the increase of $k\rho c$ during sweating, in these experiments, was expected. This increase may also be explained by increased evaporation from the moist skin surface. To differentiate between these 2 factors, India ink painted skin was exposed to a radiated energy of 100 mc/sec/cm^2 with and without being wetted with tap water. The wet skin was about 3 degrees cooler throughout 15 seconds of heating and the first 15 seconds of cooling. In this experiment there was a continuous layer of moisture superficial to the India ink, while in the sweating experiments evaporation may actually be inhibited by the overlaying India ink. Therefore, it is felt that vaporization may account for some of the effects observed, but that changes of $k\rho c$ cannot be neglected. This idea is supported by changes observed after atropine, which are mainly due to change of $k\rho c$, with little change in vaporization.

In the blood flow experiments, we have to differentiate between blood content of tissues and blood flow through the tissues. In total obstruction, blood content is decreased and blood flow is stopped. In venous congestion, blood content is increased and blood flow is practically nil. Therefore, comparing these 2 conditions, we should get the effect of blood content of the tissues. This difference was calculated in line (g) Table I. This increase in blood content increases the $k\rho c$ 14% and the equilibrium skin temperature under radiation is lowered.

The most pronounced rise of skin temperature, indicating an increase of blood flow, was obtained after release of the pressure cuff following total obstruction. However, to compare this condition of increased flow with a condition of similar blood content but without blood flow, we have to take some state between total obstruction and venous congestion, as the blood content in reactive hyperemia is surely higher than in total obstruction and lower than in venous congestion. These derived values are given in line (f) Table I. The difference between reactive hyperemia (line (c) Table I) and the derived control (line (f) Table I), yields the effect of blood flow as shown in line (h) Table I. The changes found for the effect of blood flow are in the same direction as those found previously for the effect of changes of blood content, but they are more pronounced. In these blood flow experiments the initial skin temperature and the $k\rho c$ go in opposite directions. In the short time exposure given in Fig. 6 the difference of initial temperature determines final skin temperature, while in the long time exposure of Figs. 3 and 4 the equilibrium temperature is determined by difference of $k\rho c$. We can expect a similar reversal if, instead of prolonging the time, we increase the energy of radiation.

In reactive hyperemia, the slope of the temperature increase in time is very low. We would expect a correspondingly slow fall; actually the temperature decrease with cessation of radiation is fastest in reactive hyperemia. It is assumed that this is due to heat being carried away by the blood stream.

Column (4) Table I gives the pain threshold in terms of tissue temperature, which is slightly raised over the control. This increase is considered to be due to secondary pain caused by the procedure itself and no difference between the individual procedures was found. However, the temperature increase and the time to reach the pain threshold are dependent upon the initial skin temperature.

Summary and conclusions. 1. Acetylcholine-induced sweating decreases and atropine-induced inhibition of sweating increases heating effects of radiated heat energy. Increased blood content of tissues has a slight effect and increased blood flow a more pronounced effect on lowering the heating effect. 2. The effects of sweating appear to be due partly to vaporization of water at the skin surface and partly to a change in the thermal characteristics of the skin itself. 3. Increased blood flow through the skin raises the temperature and therewith increases the heating effects of low-energy, short-time radiation. However, the

thermal characteristics of the skin are changed in a way to combat these heating effects. Therefore, the harmful effects of high energy and long term radiation are decreased. 4. Pain threshold is frequently measured as temperature elevation or as reaction time, and then it is markedly affected by changes in blood flow and rate of sweating. However, if it is studied in terms of tissue temperature, it is independent of these physiological variables.

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Antagonism of Diuretic Action of Thyroxine by Ethanol. (22442)

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Pretreatment with thyroxine causes an increase in the rate of excretion following the administration of a water load in the dog(1) and in the rat(2). Since ethanol also elicits a diuresis(3,4), these two substances were combined in an attempt to produce a diuretic response suitable for use in the biological assay of the antidiuretic hormone (ADH). Contrary to expectations, ethanol was found to abolish the diuretic action of thyroxine.

Materials and methods. Four groups of animals, each containing 12 female rats weighing approximately 200 g, were used in this experiment. The control groups and the rats that were pretreated with thyroxine were gavaged with distilled water or 8.5% ethanol in an amount equivalent to 7% of the body weight. The resulting diuresis was quantified by placing 4 rats in each urine collection cage and measuring the volume of urine from each cage at 15 minute intervals until the urine flow returned to normal. The cumulative volume of urine excreted by each group of rats was then converted to the per cent of the total water load excreted and plotted as a function of time. Pretreatment with thyroxine consisted of the daily intraperitoneal injection of 20 μg of thyroxine for 7 days prior to the gavage with distilled water or ethanol. Two groups of pretreated rats were given the water and ethanol loads intraperitoneally in order to determine if the ethanol was interfering with the absorption of the fluid from the intestine.

Results. As shown in Fig. 1, ethanol prolongs the diuresis following the administration of a water load whereas pretreatment with 20 μg of thyroxine per day results in an increased rate of excretion. Both of these treatments lead to the excretion of a greater percentage of the water load than that observed in the untreated controls. However, when pretreated rats are gavaged with ethanol, the diuretic response is similar to that observed in the rats which received no pretreatment with thyroxine. Thus the ethanol abolishes the diuretic action of the thyroxine.

Since the excretion of the water load by all 4 groups of animals is linear from 75 to 135 minutes after the gavage, the differences in the rates of excretion observed during this period were analyzed statistically using the Analysis of Variance (2 way classification) method(5). The rate of excretion of the water load by the rats pretreated with thyroxine differs significantly ($P<0.005$) from the rates exhibited by the other groups but

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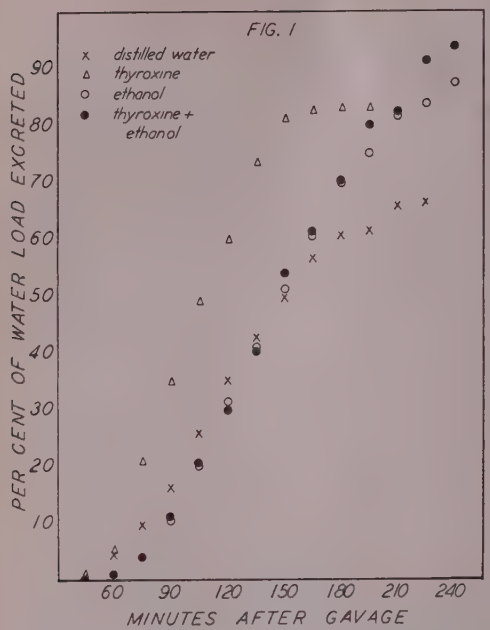

FIG. 1. Effect of ethanol, thyroxine, and combined treatment with ethanol and thyroxine on excretion of water load by the rat.

the diuretic responses of the rats gavaged with ethanol following pretreatment with thyroxine and the rats receiving only ethanol or water gavages do not differ significantly from each other.

As thyroxine is known to increase the rate of absorption of fluids from the intestines, water loads were administered intraperitoneally in order to determine whether the ethanol block occurred at this point. Since a differential rate of excretion was observed in thyroxine treated rats gavaged with distilled water and with 8.5% ethanol, the ethanol antagonism does not appear to occur in the intestine.

Available evidence indicates that the diuretic action of thyroxine is due, at least in part, to a direct action on the kidney(6) and is not dependent upon the increased BMR(7). This action of thyroxine is best explained by assuming that thyroxine increases the number of functional nephrons in the kidney(8).

Ethanol is presumed to cause a diuresis by decreasing the normal sensitivity of the supraoptico-hypophyseal mechanism to changes in the tonicity of the body fluids(3). This action is not a function of the actual amount of ethanol in the blood but rather, it is associated with an increasing concentration of ethanol in the blood. Thus a slow increase in the concentration of ethanol in the blood results in a greater diuresis than a rapid one (4).

On the basis of the accepted mechanisms by which these substances elicit a diuresis, it is difficult to explain the block of the diuretic action of thyroxine by ethanol. Since ethanol depresses the release of ADH, it is doubtful that the ethanol block of the thyroxine effect occurs at the hypophyseal level. However, it is equally difficult to explain this mechanism at the kidney level. As the ethanol block does not depress the rate of excretion below that of the untreated controls, the ethanol would have to act only on the "extra" nephrons mobilized by the thyroxine pretreatment. On the basis of the available data, it is not possible to explain this phenomenon.

Summary. Although thyroxine and ethanol possess diuretic activity when administered alone, the administration of 8.5% ethanol to rats pretreated with 20 μg of thyroxine per day for 7 days abolishes the diuretic action of the thyroxine. The ethanol block is not caused by a decrease in the rate of absorption of fluid from the intestine. The mechanism by which ethanol blocks the diuretic action of thyroxine is not apparent from the available data.

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Further Observations on Development of a Colony of Spontaneously Hypertensive Rabbits.* (22443)

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In a previous paper we described the distribution of blood pressure values and pressure characteristics of rabbits with naturally occurring hypertension(1). We concluded that there is a hereditary factor in this spontaneous hypertension and that pressure characteristics namely, lability and elevation with age, simulate those of animals in normal rabbit population. These observations were made on animals raised between Sept., 1951, and June, 1954, and included 12 families, with 2 to 4 generations each, of various strains of rabbits. We continued to interbreed these families, especially along 3 main breeding lines, *viz.*, pure New Zealand, pure Dutch and a cross between New Zealand and Dutch. From June, 1954, to June, 1955, 131 rabbits were raised to 6 months and 116 to 8 months, representing 2 additional generations. They will subsequently be referred to as "new" population. Eighty-nine and 66 rabbits which were raised to 6 and 8 months, respectively, in the 3 preceding years were described in our first paper and will be referred to as "old" population. This paper presents the distribution of blood pressures among recent members of the colony, *i.e.*, the "new" population, and compares it to that of their progenitors, *i.e.*, the "old" population. In addition, further findings on blood pressure characteristics are described for rabbits selected from the colony and for closer study.

Methods. Rabbits have a natural life span of 3 to 7 years and rapid growth the first 2 months of life; they may be considered adults at 4 to 6 months. When rabbits are 3 to 4 months of age, we begin taking routine pressures at least twice a month up to 8 months of age. Unless animals are used as breeders or for research purposes, they are sacrificed at 10 to 12 months because of insufficient housing facilities. Other animals die of intercurrent diseases at various ages. Autopsies are performed whenever possible, and the findings are reported below. Routine auscultatory pressures are taken from the abdominal aorta using a sphygmomanometer cuff wrapped around the abdomen. This method was described in detail by McGregor(2). The animal is placed on the board, and 4 systolic and diastolic readings are made within 2 minutes. Another set of 4 readings is taken 5 minutes later. Four successive readings seldom vary more than 5 to 10 mm Hg. The average for each set of 4 readings is recorded along with heart rate, body weight, and condition of the animal. Averages of such sets of readings taken 5 minutes apart are often the same but may vary 10 to 15 mm Hg in either direction. The same results were reported by McGregor(2). When animals were brought into the laboratory, blood pressures were taken by the cuff method and, in addition, by direct femoral artery puncture with a Statham pressure transducer; the latter procedure and its correlation with cuff systolic pressures has been described earlier (1).

Results and Discussion. We selected from routine pressure readings the highest systolic pressure recorded during each of three 2 month periods. Table I presents the mean value of this highest systolic pressure of each indicated age group for both "new" and "old" populations. In addition, Table I shows the number, and per cent, of animals with systolic pressures above, and below, 150 mm Hg. The latter is the systolic value we consider to be the upper limit of normal(1). Fig. 1 shows complete frequency distribution of highest systolic pressure at 3-4 and 7-8 months of age in the "old" and "new" populations.

Table I and Fig. 1 point out that as the 2

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TABLE I. Distribution of Highest Systolic Blood Pressure† Obtained by Abdominal Cuff Method in a Spontaneously Hypertensive Rabbit Colony.

	"New" population					
Age (mo)	3–4		5–6		7–8	
Total No.*	125		131		116	
Systolic pressure (mm Hg)	111–150	151–200	111–150	151–200	111–150	151–200
No. rabbits	61	64	55	76	34	82
% total No.	49	51	42	58	29	71
Mean ± S.D.	151 ± 17		155 ± 15		162 ± 18	
	"Old" population					
Age (mo)	3–4		5–6		7–8	
Total No.*	84		89		66	
Systolic pressure (mm Hg)	111–150	151–200	111–150	151–200	111–150	151–200
No. rabbits	57	27	35	54	18	48
% total No.	68	32	39	61	27	73
Mean ± S.D.	144 ± 15		157 ± 16		158 ± 16	

* At 3-4 mo of age, some rabbits are too small to use for abdominal cuff determinations which accounts for fewer animals in this age group than at 5-6 mo. The reason less animals were available at 7-8 mo is that some died from intercurrent diseases.

Calculation of Fisher's "t" values showed that the means at 4 and 8 mo for both "new" and "old" populations are significantly different ($p > .001$) and means at 6 and 8 mo for "new" population are significantly different ($p > .01$). The difference in 4 mo means between the "old" and "new" populations is significantly different ($p > .001$).

† This refers to the set of 4 successive readings having highest average systolic pressure.

populations grew older their systolic pressures increased, and the "new" population had significantly higher pressures at 3-4 months of age, than their progenitors. Although mean systolic pressure at 8 months was somewhat higher in the "new" population, the difference between the two was not significant.

The "old" population did not show a pressure difference between the two sexes whereas the latest offspring did. The data in Table I for "new" population has been separated according to sex, and the results appear in Table II. Males had mean values for systolic pressure significantly higher than females, in all 3 age groups. Moreover, mean systolic pressure of "new" males is higher than in either male or female progenitors, whereas the mean systolic pressure of "new" females is lower than that of their progenitors. Other workers studying the effect of age on blood pressure in fowls found that females had lower pressures than males at both 10 and 50 months of age(3). On the other hand, no difference was found between sexes in Wistar rats known to develop high blood pressure with advancing age(4). In humans rate of rise in pressure with advancing age differs in male and female(5).

Diastolic pressure can be obtained with an

TABLE II. Mean Values and Distribution of Highest Systolic Pressure* in "New" Male and Female Members of a Spontaneously Hypertensive Rabbit Colony.

	♂			♀		
No. of animals	62	68	63	63	63	53
Age in mo	3–4	5–6	7–8	3–4	5–6	7–8
Mean systolic pressure (mm Hg)	156	162	169	149	149	152
% animals above 150 mm Hg systolic	60	75	86	43	40	53

* This refers to set of 4 successive readings having highest avg systolic pressure.

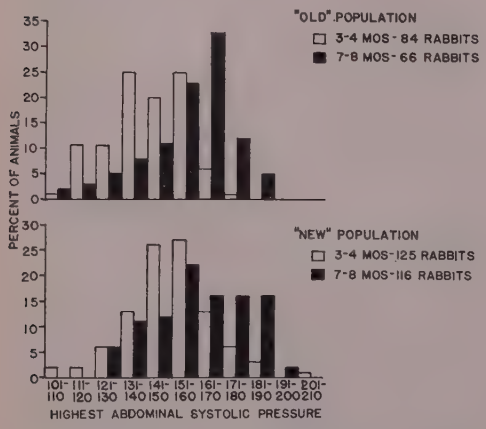

FIG. 1. Distribution of highest systolic pressure at 4 and 8 months in "old" and "new" populations.

TABLE III. Femoral Artery Pressures in Spontaneously Hypertensive* and Normotensive Stock Rabbits.

	No. rabbits	No. determinations	Systolic Range	Systolic Avg	Diastolic Range	Diastolic Avg
Spontaneously hypertensives	25	35	131–180	151	78–124	97
Normals	10	11	121–136	128	83– 92	87

* From both "old" and "new" populations.

accuracy of ± 10 mm Hg, at best, by the abdominal cuff method. For this reason direct femoral artery punctures were made on a group of spontaneously hypertensive rabbits using normotensive stock animals as controls. Table III presents findings with accompanying systolic pressures. Spontaneously hypertensive rabbits had average diastolic elevation of 10 mm Hg above that of normals. Individual values indicated that small rabbits (Dutch breed), known to mature sooner than New Zealands, had higher diastolic pressures than the others.

One of the pressure characteristics in spontaneously hypertensive rabbits is marked lability, *i.e.*, a noticeable pressure variation from month to month. We brought animals into the laboratory to study this factor more closely. For a period of several months, daily or twice weekly, auscultatory pressure readings were taken in the usual manner. The pressure value recorded each time was the average of 4 successive readings which agreed within 5 to 10 mm Hg of each other. Despite the fact that an animal was tied down on a board in the unanesthetized state, the day to day fluctuation in average systolic pressure was only 10 to 15 mm Hg with an occasional fluctuation exceeding 25 mm Hg. This was true if pressures were taken at the same time and under the same conditions each day. However, during several weeks the average systolic pressure might gradually shift 20 to 30 mm Hg and stabilize at some new level. It is the extreme values of such pressure shifts which one may obtain by chance when only occasional pressures are taken, *e.g.*, twice a month, and which accounts for noticeable lability of pressure. Normotensive rabbits, under the same conditions, show the same magnitude and type of blood pressure fluctuations. McGregor found that daily readings obtained over a 21 day period had extreme fluctuations of 20 mm Hg(2).

Direct arterial measurement is the only accurate means of obtaining moment to moment pressure variations. Fig. 2 shows representative intraarterial tracings from the carotid arteries of a normotensive stock rabbit and two spontaneously hypertensive rabbits taken while they were supine and unanesthetized. Spontaneously hypertensive rabbits showed 2 characteristic fluctuations of pressure—small pressure fluctuations of about 1 second duration and relatively large fluctuations, 15 mm Hg of 4 to 7 seconds duration. The duration of small fluctuations corresponds to length of a single respiratory cycle, the latter being 1 to 2 seconds in rabbits. The 4 to 7 second duration waves resemble Traube-Hering waves of rabbits both in duration and magnitude(6). The normal animal's record in Fig. 2 shows shallow waves 3 to 4 seconds in duration; the 1 second duration waves are not present. Research on Traube-Hering waves has been carried out in partially curarized or anesthetized open-chest preparations. Such studies led to the conclusion that CO_2 concentration in vasomotor and respiratory centers affects duration of Traube-Hering waves while peripheral vascular tone determines their magnitude(6,7). The records in Fig. 2 indicate that Traube-Hering waves are present under normal physiologic conditions in rabbits. Since spontaneously hypertensive rabbits probably have increased vascular tone, this would account for their having greater wave magnitude than the normotensive animal.

Kidney changes were found in 36 of 86 autopsies of our colony animals, sacrificed or dead from intercurrent disease; in 50 animals the kidneys appeared normal. In the majority renal changes consisted of tiny, congested areas, 1-2 mm in diameter and depressed

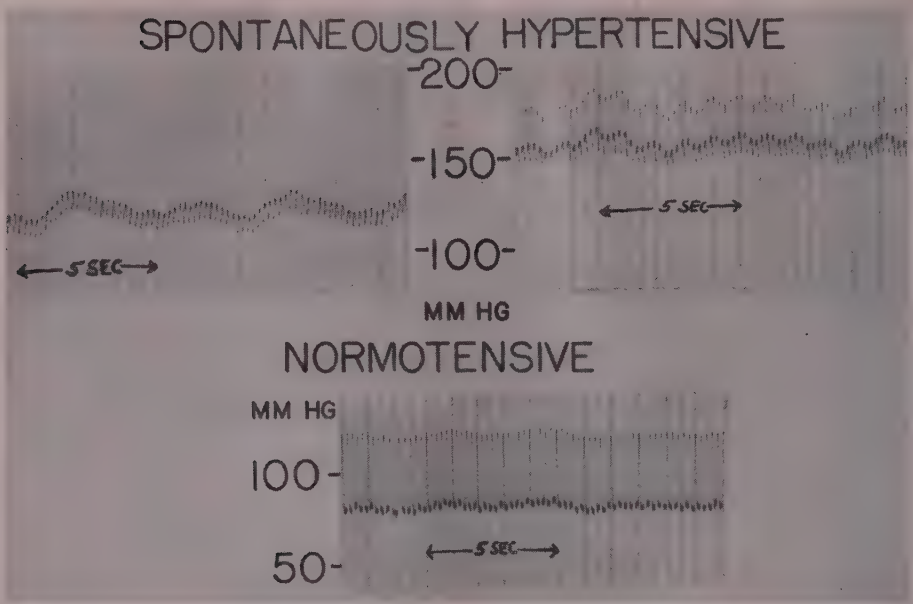

FIG. 2. Recordings from the carotid arteries of two spontaneously hypertensive and one normotensive rabbit showing Traube-Hering waves (see text).

about 0.5 mm, scattered over the surface of normal sized kidneys. The cut surface showed mild tissue striation of the medullary region. In others the kidney surface was knobby, and the tissue tough to cut. Marked medullary striation and a narrowed cortex was seen. Twenty-eight of the 36 abnormal kidneys were examined microscopically, and the findings presented in Table IV. Eighteen of the 28 animals (64%) had been hypertensive and 10 (36%) normotensive during life. These percentages are representative of pressure distribution in our colony (Table I). The significant fact shown in Table IV is that interstitial nephritis was found in normotensive animals. Although in this series none of the normotensives had the more marked degree of nephritis, an earlier report from our laboratory showed marked interstitial nephritis to be present in animals with normal blood pressure(8). Lesions, described in detail in that report, are identical to those found in these colony rabbits. The same paper also noted that if normal laboratory animals were subjected to laparotomy or intravenous procedures, the incidence of interstitial nephritis reached 66.6%. (*Staphylococcus albus* was cultured from some of the kidneys implicating the hematogenous route.) Twenty-four of our 36 cases were known to have had infections, surgery or unsterile intramuscular injections during life.

Presence of interstitial nephritis in normotensive animals indicates that these renal changes are incidental and not a causative

TABLE IV. Microscopic Findings in 18 Hypertensive and 10 Normotensive Animals Which Showed Renal Pathology at Gross Autopsy Examination.

	Hypertensive		Normotensive	
	No.	%	No.	%
Normal	4	22.2	1	10
Interstitial nephritis:*				
Minimal to mild	8	44.4	9	90
Moderate to marked	3	16.6		
Moderate + amyloidosis	1	5.6		
Unilateral ascending pyelonephritis†	1	5.6		
Amyloidosis	1	5.6		
Totals	18	100.	10	100

* The term, interstitial nephritis, is synonymous with pyelonephritis arising by the hematogenous route. Degree of interstitial nephritis based on scars per low power field: minimal, less than 1; mild, 1; moderate, 2; marked, 3 or more.

† Associated with unilateral hydronephrosis.

factor in production of spontaneous hypertension.

Eleven of the 50 normal appearing kidneys were examined microscopically. One of these had a focal glomerulitis and came from a normotensive animal. The other 10 were normal microscopically and were from hypertensive animals.

Calcification of large and medium size arteries was found in the first family of rabbits raised in our colony, but this condition died out the following generation and has only shown up occasionally in other animals. Enlarged hearts have not been found at autopsies. One probable reason is that spontaneous hypertension is a relatively mild labile type of hypertension and, in addition, autopsies have been done in the early part of the animal's life span.

Summary. (1) Two new generations of spontaneously hypertensive rabbits showed about the same incidence of elevated systolic pressures as their progenitors at 6 and 8 months of age, 58 and 71% respectively. At 4 months, mean systolic pressure in the 2 new generations was significantly higher than in their progenitors at same age. (2) Male rabbits of the new generations in all 3 age groups had higher mean systolic values than females and than their male or female progenitors. (3) Direct arterial pressure measurements in a group of spontaneously hypertensive rabbits showed average diastolic pressure to be 10 mm Hg higher and systolic pressure 30 mm Hg higher than in normotensive stock rabbits. (4) Direct arterial recordings from unanesthetized animals demonstrated spontaneously hypertensive rabbits to have larger Traube-Hering waves than normotensives. (5) The presence of interstitial nephritis in normotensive as well as spontaneously hypertensive animals precludes it as an etiological factor in the development of spontaneous hypertension.

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Effects of Corticosterone, Cortisone and Hydrocortisone on Fat Metabolism in the Chick. (22444)

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The influence of glucocorticoids on fat metabolism is well documented in many species(1-5). There are, however, several published reports which indicate that there are some qualitative differences exhibited by these hormones on lipid metabolism(3,6). This is particularly interesting since these steroids are considered to be qualitatively similar to their physiological effects and any differences are primarily quantitative(7).

It was therefore of interest to determine the effects of crystalline adrenal cortical hormones on fat metabolism in the chick. Accordingly, the ability of corticosterone (compound B), cortisone (compound E), and hydrocortisone (compound F) to produce changes in fat deposition in the chick was studied.

Materials and methods. Single-comb white leghorn cockerels were used throughout these

TABLE I. Effects of Compounds B, E and F on Body Weight Gain, Adrenal Weight, and Liver Weight and Lipid Content in the Chick.

No.	Treatment	Body wt gain (g)	Adrenal wt Actual (mg)	Adrenal wt mg %	Liver wt Actual (mg)	Liver wt % body wt	% liver lipids	Liver color
			Exp. 1					
8	Controls	155	60.0	14.1	9.4	2.30	1.90	Red
9	1.5 mg cortisone	133	53.1	13.2	9.9	2.58	2.11	"
10	1.5 corticosterone	58	44.8	14.0	15.9	5.01	17.40	Yellow
8	1 hydrocortisone	36	39.1	13.7	17.1	5.79	19.96	"
			Exp. 2					
10	Controls	111			10.2	2.40	2.31	Red
10	10 mg cortisone*	30			7.9	2.30	2.60	"

* Acetate.

experiments and allowed to eat (Vitality chick grower) and drink (tap water) *ad libitum* during the course of treatment. The animals were 28 days of age at the beginning of the experiment. On the day of the first injection all birds were leg-banded, weighed and placed into groups of equal average body weight. The steroids used were suspended in a vehicle consisting of 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol and 0.9% sodium chloride. All injections were made subcutaneously in the volume of 0.2 cc once a day for 10 days. Animals were sacrificed approximately 24 hours following the last injection. At autopsy the body, adrenal and liver weights were determined. The livers were frozen immediately after removal and stored in a deepfreeze until fat determinations could be made. The fat content was determined by the method of Horwitz(8). Two livers from each group were fixed in 10% neutral formalin for histochemical studies on fat content. Frozen sections were stained with Sudan 4 and Harris' hematoxylin in order to determine the distribution and quantity of fat present. The birds were also examined grossly for changes in distribution of body fat.

Results. The effects of cortisone, corticosterone and hydrocortisone on body growth, adrenal weight and liver weight and liver fat content are shown in Table I. It is seen from these data that corticosterone at 1.5 mg per day and hydrocortisone at 1 mg per day produced a marked increase in liver weights and liver fat content. Cortisone, however, did not influence the weight or fat content of the liver at a dosage as high as 10 mg per day (Exp. 2).

It was also observed that compounds B and F caused considerable subcutaneous fat deposition and an increase in fat around the viscera. The most obvious foci of increased fat deposition was around the auricles of the heart, in the cloacal region, the strip of fat along the sternum, and the fat depot of the neck area. In contrast to these effects, cortisone produced no noticeable changes in fat deposition at either dosage level used.

The body growth of the B (1.5 mg) and F (1.0 mg) treated animals was considerably inhibited, whereas in the cortisone treated birds body growth was suppressed only slightly at 1.5 mg per day while at 10 mg per day there was a considerable inhibition of body weight. The adrenal weights, as related to body weights, were not inhibited by any of the treatments, whereas the actual adrenal weights, if not corrected for change in body weight, were decreased in the steroid treated animals.

Frozen liver sections stained with Sudan 4 confirmed the results obtained by extraction of liver fat. In general these slides showed essentially no sudanophilic material in the livers of the control or cortisone treated animals, whereas the livers of the F and B treated birds possess large quantities of sudanophilic substances. It is noteworthy that the fat was accumulated around the blood vessels in the B and F treated animals and that there was no apparent degeneration of the liver cells.

Discussion. The results reported in this

paper extend and confirm those reported by Stamler(3) who recently found that hydrocortisone but not cortisone influenced lipid metabolism in the chick. Stamler did not mention, however, whether or not there was any increase in carcass, visceral or liver fat in the hydrocortisone treated animals. The fact that hydrocortisone and corticosterone produced considerable fat deposition in the liver and on the carcass and viscera, while cortisone did not affect fat deposition, was somewhat surprising. In the mammal, compounds E and F are qualitatively alike as to their effects on fat metabolism. It has been reported that both E and F produce lipemia in the rabbit(9) and an increase in total body fat and lipemia in the human(10). The results reported here, therefore, show that a striking species difference exists between the mammal and the bird as to the activities of compounds B, E, and F on fat metabolism.

The catabolic effect of the glucocorticoids as shown by negative nitrogen balance and inhibition of body growth is well known(7). The relative effectiveness of these three steroids on protein metabolism in mammals in descending order of potency is as follows: hydrocortisone, cortisone, and corticosterone(7). It is noteworthy that in the chick hydrocortisone and corticosterone are considerably more effective in inhibiting body growth than cortisone. The fact that cortisone inhibited body growth at the higher dose indicates that its failure to induce changes in fat distribution was not due to a complete lack of physiological effects. The fact that hydrocortisone inhibited body growth as well as producing changes in fat metabolism while cortisone only inhibited growth suggests that a qualitative difference exists between these two hormones in their effects on fat metabolism in the chick.

The evidence that hydrocortisone, corticosterone and cortisone did not influence the relative adrenal weights of the chick is of interest and in agreement with earlier reports(3,11). In the rat these 3 compounds inhibit the pituitary-adrenal axis as indicated by a decrease in adrenal weight(7). The reason for this apparent functional discrepancy in the pituitary-adrenal axis between birds and mammals is not clear at this time but it has been suggested by earlier workers(11,12) that the bird adrenal may exhibit some activity independent of the pituitary.

Summary. (1) Hydrocortisone and corticosterone caused an increase in liver, visceral and carcass fat in the chick while cortisone was without effect. (2) The relative effectiveness of these 3 steroids on body growth inhibition in the growing chick is in the following order: hydrocortisone, corticosterone and cortisone. (3) None of these 3 hormones caused an inhibition of relative adrenal weights.

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Fate of Shed Mast Cell Granules.* (22445)

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Numerous investigations concerning the role of the mast cell in the physiology of the connective tissue have been stimulated following the observations that both heparin(1) and histamine(2) are concentrated in the cytoplasmic granules of this cell. More recently it has been reported that 5 hydroxy-tryptamine is also a constituent of mast cell granules(3). Ehrlich, who described the mast cell, observed that it appeared as an overnourished cell whose cytoplasm was well-filled with metachromatic granules and suggested that it may function as a storage cell of the connective tissue(4).

It has been reported that when loose connective tissue is exposed to any of various noxious stimuli, the mast cells therein release or shed their granules into the surrounding ground substance(5). In view of the proposed constituents of the mast cell(1-3), it has been of interest to ascertain the fate of these shed granules. In the following study it was observed that within 15 minutes to 1 hour after degranulation, metachromatic granules appeared within the cytoplasm of the surrounding fibroblasts. Further investigation showed that these fibroblastic granules were actually shed mast cell granules which had been taken into the cytoplasm of the fibroblasts.

Materials and methods. Injected materials. Materials for injection were suspended in sterile, pyrogen-free isotonic saline. 1. Mast cell granules were obtained by a method developed in this laboratory(6), which is as follows: sheets of loose subcutaneous connective tissue were stripped from the skins of freshly killed mice by applying the connective tissue surface of the skin to a freezing surface (stainless steel over dry ice) and then rapidly pulling the skin away, leaving the connective tissue as a frozen sheet. This tissue was then scraped into an isotonic sucrose solution and shaken over night with glass beads in a cold room (4°C). The mast cell granules were isolated from the mixture by differential centrifugation. Fig. 1 shows the nature of this granule preparation. 2. Radioactive mast cell granules were isolated from the connective tissue of 35 to 40 female CBA mice (12 to 16 months old) by the above technic. These animals had been injected intraperitoneally 48 hours previously with 0.1 cc of a carrier-free solution of $Na_2S^{35}O_4$‡ containing approximately 250 μc activity. Radioautographic analysis of tissue spreads at intervals after injection, obtained from similarly prepared mice, has shown that the highest relative concentration of radioactivity in the mast cells could be obtained approximately 48 hours after injection of the radioactive solution (Fig. 2). This is in agreement with the observations of Jorpes, *et al.*(7) as well as with those of Asboe-Hansen(8). Preparations of isolated granules (Fig. 3) were washed 2 times with isotonic sucrose to free the preparation from any excess radioactivity not bound to granules, then resuspended with isotonic saline for injection. *Tissue sampling.* CBA mice, 8 to 10 weeks old, were injected subcutaneously on the dorsal surface with 1.0 cc of air to form a bubble of air in the loose connective tissue (air pouch). The details of this method will be described elsewhere. Treatment and sampling of the tissue were carried out routinely in the following manner: test substances contained in a volume of 0.2 cc were injected into the pouch. After sacrifice of the animal at the appropriate interval following injection, the pouch was dissected free of the surrounding tissues and a section

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‡ $Na_2S^{35}O_4$ was obtained from Oak Ridge, Tenn.

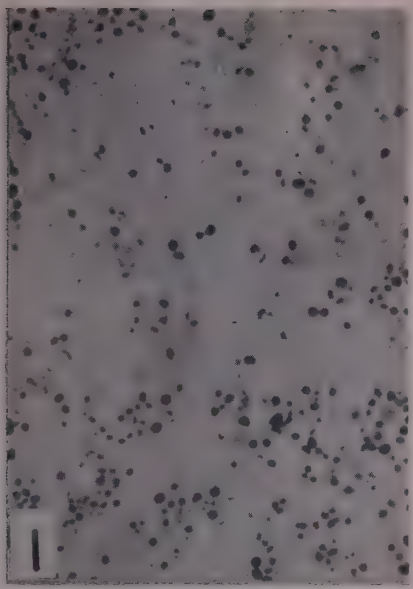


FIG. 1. Mast cell granules following extraction from mouse loose connective tissue. Stained with alcoholic solution of Azure A. (× 960)

of the enclosed connective tissue bubble rapidly removed, spread on a glass microscope slide, air-dried, and stained with May-Gruenwald Giemsa in the usual manner(9). Similar preparations were stained with an alcoholic solution of Azure A for comparison. *Radioautographic technics.* Contact radioautograms of connective tissue spreads were prepared by apposition to 10 μ thick NTB, nuclear track plates (Eastman Kodak Co., Rochester, N. Y.). The radioautogram was superimposed upon the corresponding connective tissue spread and examined microscopically. A more detailed examination of the radioautogram and tissue spread was made by taking photomicrographs from corresponding areas of tissue spread and radioautogram (Fig. 2 and 9). Detailed radioautograms of the injection material (radioactive granules spread on glass microscope slides) were prepared with Eastman Kodak's permeable base stripping film. The technic used is a modification of a method described by Arnold and Jee(10). Due to the extensive washings of the tissue preparation, the granules were swollen (Fig. 3) and had a reduced affinity for the stain. Control preparations reacted

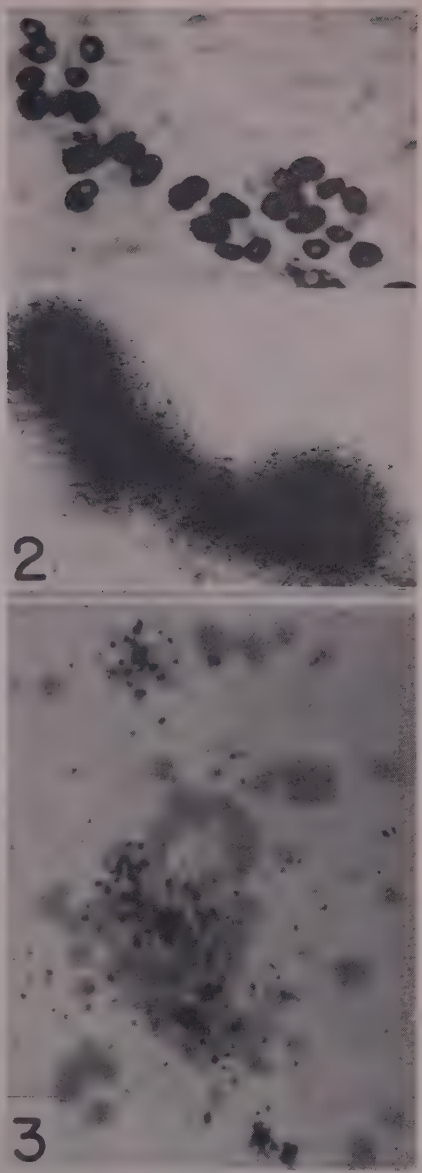
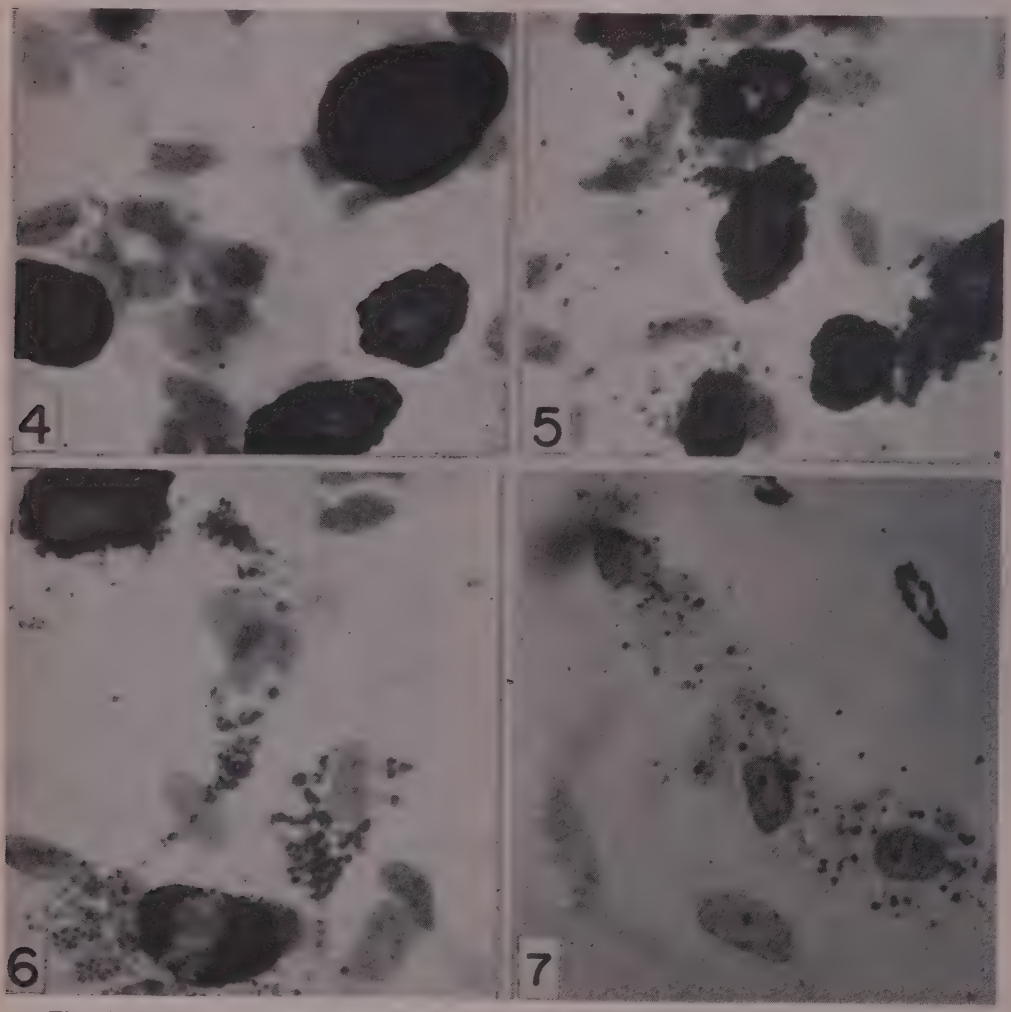


FIG. 2. Loose connective tissue spread (upper) from mouse 48 hr after inj. of $Na_2S^{35}O_4$, and contact radioautogram (lower) of accumulated radioactivity in the mast cells. Note conformity of mast cell distribution with darkened area of film. (× 500)

FIG. 3. Strip film radioautogram of mast cell granules isolated from subcut. loose connective tissue of mice inj. with $Na_2S^{35}O_4$ 48 hr previously. The granules are somewhat swollen and weakly staining following radioautographic procedures. However, note that the radioactivity (black dots) is concentrated with the granules. (× 1350)

to the stain as those in Fig. 1. However, it is apparent that the radioactivity was bound to the mast cell granules.

Results. Following the local injection of various substances (*e.g.*, histamine, gelatin, agar-agar, protamine, saline, etc.), the mast cells, which are normally quite intact and filled with granules (Fig. 4) can be seen to degranulate and shed their granules into the surrounding ground substance (Fig. 5). The number of mast cells exhibiting this reaction is generally related to the nature of the material injected; that is, the injection of air or saline will stimulate a small reaction, whereas



Figs. 4-7. Loose subcutaneous connective tissue spreads. Stained with May-Gruenwald Giemsa. (× 850)

FIG. 4. Mast cells prior to exposure to noxious stimuli.

FIG. 5. Non-specific degranulation of mast cells in response to stimuli (0.1 mg histamine).

FIG. 6. Fibroblastic uptake of shed mast cell granules by cells proximal to degranulating mast cells. Note the concentration of granules in the poorly staining cytoplasm of fibroblasts at center of microphotograph.

FIG. 7. Fibroblastic digestion of ingested mast cell granules after degranulation of mast cells 2 hr earlier. The cytoplasm exhibits varied degrees of metachromasia as the granules undergo dissolution.

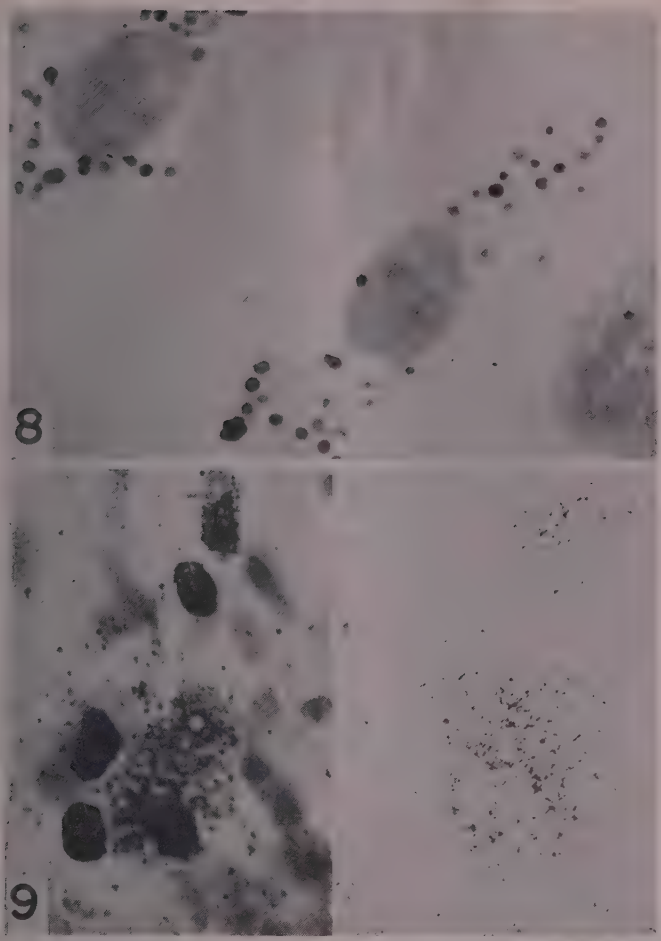


FIG. 8. Loose subcut. connective tissue spread from mouse inj. subcut. 2 hr previously with preparation of isolated mast cell granules. Observe that granules are confined within cytoplasm of cell. Stained with May-Gruenwald Giemsa. (× 1250)

FIG. 9. Loose connective tissue spread (left) and contact radioautogram (right) from mouse 1 hr following inj. of preparation of isolated S^{35}-labeled mast cell granules. Inj. granules at center show retention of radioactivity, whereas little to no radioactivity is associated with mast cells to left and above center. Note presence of "newly granulating" cell at upper border in comparison to relative lack of activity associated with nearby mast cell. Stained with May-Gruenwald Giemsa. (× 500)

agar-agar or histamine provokes a rather extensive amount of degranulation. Injection of a mild antigen (horse serum) into the connective tissue of sensitized animals produces a rapid and marked degranulation of the exposed mast cells, whereas the same antigen injected into a non-sensitized animal elicits a comparatively small response(11). It has therefore appeared unlikely to us that degranulation is an artifact, as recently suggested by Devitt(12). In a series of experimental observations in this laboratory over a period of years, it has become apparent that degranulation is followed by the appearance of granules in the fibroblasts of the local area (Fig. 6). This is evident within 15 minutes to 1 hour after degranulation has been initiated. During the next 1 to 2 hours, the presence of granules in fibroblasts tends to decrease and many of the fibroblasts have a

diffuse metachromasia in the cytoplasm and/or contain granules in various stages of dissolution (Fig. 7). This suggests that many of the fibroblasts are able to digest this material.

Isolated Granules. To confirm these observations, mast cell granules isolated from mouse connective tissue relatively free from other cellular elements (Fig. 1) were resuspended in isotonic saline and aliquots injected into mice subcutaneously. At intervals of 15, 30, and 60 minutes, 2, 3, 4, 5 and 6 hours, tissues were obtained from at least 4 animals and prepared for microscopic observation. Only those tissue areas containing patches of injected granules and relatively free of mast cells were examined, and it was observed that, within 15 minutes after injection, metachromatic material as well as granules appeared in the cytoplasm of fibroblasts. The granule uptake appeared to reach its maximum within the first to second hour following injection. By the fourth hour the cytoplasmic granules had largely disappeared. Fig. 8 shows an area of a tissue sample 2 hours after granule injection in which the cytoplasm of the fibroblasts contains typical mast cell granules. The time necessary for granule digestion is probably much less than our observations would suggest, since in any one cell there is usually evidence of granule breakdown coexistent with normal appearing granules.

Radioactive Mast Cell Granules. To test these observations further, mice were injected subcutaneously with aliquots of an S^{35}-labeled granule preparation. Samples of loose connective tissue were removed from the mice 1 hour after the injection with radioactive granules and prepared for contact radioautography. In Fig. 9 the labeled granules can be seen in the tissue with a distribution of radioactivity in fair agreement with granule distribution. It should be noted that the intact mast cells show no evidence of accumulation of radioactivity, while there is an apparent formation of a "new" mast cell resulting from the fibroblastic uptake of radioactive granules.

Discussion. Connective tissue is a primary site of inflammation and serves as a mediator between the parenchymal cells and the blood-vascular system throughout the body. Probably the earliest obvious change occurring in the loose connective tissue following stimulation by any of a number of noxious agents is the degranulation of the local mast cells. It has been demonstrated that, following degranulation, these granules can be taken into the cytoplasm of the local fibroblasts, where they are digested. In essence, this phenomenon is a turnover of mucopolysaccharide in the connective tissue. It is probable that this is but a single aspect of a more general fibroblastic participation in the catabolism of ground substance mucopolysaccharides.

This fibroblastic function, unlike classical phagocytosis, is not generalized with respect to the particle. When connective tissue is exposed to an inoculum of young staphylococci, it shows a selective fibroblastic uptake of granules from degranulated mast cells in the tissue, rather than of the bacteria. Since this cellular phenomenon has been demonstrated principally with the heparin-containing mast cell granules or polysaccharides of large molecular weight of (or resembling those of) tissue origin, it has been termed "micellophagosis"(13) and implies the fibroblastic uptake of polysaccharides or polysaccharide-bound substances.

In view of the biologically active nature of some of the proposed constituents of mast cell granules (*i.e.*, heparin(1), histamine(2), and 5 hydroxytryptamine(3)), this fibroblastic function readily suggests itself as a protective mechanism for the maintenance of connective tissue function. Tissue injury initiates the release of a factor or factors (*e.g.*, histamine, etc.), which can induce the shedding of mast cell granules. The intracellular granules may serve to fix low concentrations of noxious amines in a manner similar to their binding (vital staining) with the amine, toluidin blue. Larger concentrations of the amines may injure the mast cell and thereby stimulate the release of granules into the ground substance, where the acidic polysaccharide (heparin) constituent of the granules may continue to bind additional amines before ingestion by local fibroblasts. The release of histamine from mast cell granules by the phenyl alkyl-

amine, 48/80(14), and the binding of heparin by this substance(15) suggest that this granule constituent may exchange histamine for various other amines, thereby forming complexes with the latter substances. In any event, ingestion and subsequent detoxification of this material by fibroblasts would tend to reduce the stimuli for the inflammatory response. Under physiological circumstances, noxious amines arising as the result of metabolism and/or cell death could be dealt with in these manners and the production of inflammatory lesions avoided(16).

Summary. It has been observed that following mild trauma to the skin of mice, degranulation of mast cells occurs in the subcutaneous connective tissue and is followed by the appearance of metachromatic granules within the cytoplasm of surrounding fibroblasts. Investigation of this phenomenon by subcutaneous injection of isolated mast cell granules showed that fibroblasts began to ingest the granules within 15 minutes after injection. Granulation appeared to be maximal within one to two hours and digestion of granules was apparent within four hours. These observations on the fibroblastic uptake of mast cell granules were confirmed by using S^{35}-labeled mast cell granules for injection. It is suggested that this phenomenon represents one aspect of local detoxification in the tissues.

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Effect of Total Body X-Irradiation on the Parakeet.* (22446)

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The biological effect of total body x-irradiation on vertebrates has been investigated chiefly in mammals; a few data have also been reported on fishes, amphibians, and reptiles(1). Although birds retain many characteristics of reptiles, they differ from the latter in being homeothermal and having a metabolic rate akin to that of mammals. Nevertheless, relatively few radiation studies have been conducted on birds, and most of these have employed the chicken(2-5). To provide a broader base for comparing the response of birds with other classes of vertebrates we have

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carried out this study on the shell parakeet, *Melopsittacus undulatus*.

Material and methods. The 336 parakeets used in these experiments were obtained from commercial dealers and the previous genetic history of the birds is unknown, except that no inbred strains were represented. Both sexes were used, but there was a slight excess of males; in age the birds ranged from 3 to 18 months. Before and after irradiation they were kept in roomy cages, usually 15 birds in a 1 x 2 x 3 foot cage. They were fed a mixture of equal parts millet and canary seed *ad libitum*. Once weekly water-soaked oats mixed with cod liver oil as well as carrot greens were provided. Water and oyster shell grit were always available. At the time of irradiation each bird was confined in a 4 x 4 x 10 cm compartment of a plexiglass box. The box measured 20 x 20 x 4 cm and contained 10 such compartments arranged in 2 parallel rows of 5 each. The outer walls and partitions were perforated by holes 5 mm in diameter and 10 mm apart. During irradiation, when 4 boxes were usually stacked one above the other, they were separated by a 1 cm space and ventilated with a gentle current of air from an electric fan; the room was air conditioned. The boxes rested on a turntable that rotated at about 1 rpm. The motion was transmitted by a lucite rod 5 cm in diameter that elevated the turntable 65 cm above the motor housing. To eliminate the influence of any dose difference in the vertical axis the order of stacking the boxes was systematically changed during irradiation. The containers were removed from the stack at different times depending on the dosage to be delivered to any group of 10 birds. To exclude the possibility that close confinement in the boxes during irradiation might affect the mortality, several experiments were carried out in which only 3-5 birds were kept in a commercial plastic bird cage as large as all 4 of the experimental cages combined. No difference in radiation response was noted. A constant potential Quadrocondex therapy machine operated at 250 KV and 15 ma served as the x-ray source. A filter of 0.5 mm copper and 1.0 mm aluminum was located 7 cm from the anode. An additional compensating filter of 3 copper sheets whose contours followed the isodose curves, was placed 12 cm from the anode. This filter reduced a dose variation of 8% within the field occupied by the boxes to 4%. The half value layer in the center of the field was 1.8 mm copper. The boxes were 1 meter from the anode; a Siemens integrating dosimeter was located at the level of the stack center, 2 meters from the anode and 40 cm from the axis of the x-ray beam. By previous calibration with a 100 r Victoreen chamber this dosimeter was used to measure accurately the total dose received by the birds at any time during the period of irradiation. The dose rate delivered at the boxes was approximately 22 r/min.

TABLE I. Survival after Total Body X-Irradiation.

Dose (roentgens)	No. of birds	No. dead in 30 days	Avg time of death (days)	Birds surviving 30 days (%)
500	12	1	13.0	92.5
1250	33	3	12.3	90.9
1500	88	24	12.9	72.7
1750	47	21	10.3	55.3
2000	77	54	10.5	30.0
2250	18	13	13.3	27.7
2500	30	24	12.6	20.0
2750	20	20	13.8	0.0
3000	24	22	11.5	8.3

Results. The parakeets proved to be surprisingly resistant to total body x-irradiation. The doses employed ranged from 500 to 3000 r; even at the highest dose an occasional bird survived (Table I). Approximately 55% of birds live for 30 days after exposure to 1750 r; at 2000 r the survival is only 30%. The LD 50 30 was calculated to be 1800 $\pm$ 75 r. Mortality, regardless of the dose, was highest during the second week after irradiation. Birds that did not die in the first 3 weeks usually survived indefinitely; many are still under observation 1 to 2 years after irradiation.

Radiation Sickness. During the first 5 days after irradiation the birds showed little change in activity or behavior. At the end of this period, however, those that had received a large dose of radiation became quiet, often sitting on their perch with eyes closed and feathers ruffled. They stopped eating and as

TABLE II. Effect of 2000 r Total Body Irradiation on Peripheral Blood Count.

Sex	Days past radiation	RBC count* Before radiation	RBC count* After radiation	% RBC surviving	WBC count Before radiation	WBC count After radiation	% WBC surviving
♀	2	2.95	2.32	78	24,000	14,800	62.0
♂	2	4.12	2.97	72	26,000	8,880	34.1
♀	4	3.33	2.24	67	26,660	9,760	36.6
♂	4	4.44	2.49	56	29,320	9,320	31.8
♀	6	3.01	2.49	82	12,220	1,540	12.6
♂	6	4.18	2.05	48	18,660	5,320	28.5
♂	8	3.99	2.42	60	17,540	1,320	7.5
♂	8	3.87	2.37	61	9,100	1,330	14.5
♀	11	4.31	3.26	75	29,100	1,100	3.7
♀	11	4.12	2.28	55	46,660	880	1.8
♀	13	4.37	2.75	62	13,320	100	.7
♀	13	4.36	2.36	54	30,440	3,540	11.6
♀	15	4.05	2.26	55	42,220	9,100	21.5
♂	15	4.79	2.54	53	27,760	220	.8
♀	19	4.80	1.75	36	38,880	3,100	7.9
♂	19	3.42	1.09	31	40,220	2,880	7.1
♀	22	4.23	2.14	50	25,320	2,376	9.3
♂	22	4.00	3.17	79	28,880	2,132	7.4
♀	26	3.71	2.18	57	24,440	5,540	22.6
♀	26	3.81	2.63	68	28,880	4,220	14.6

* Expressed in millions.

they became weaker would leave the perch to rest on the bottom of the cage. The excrement, which in birds is a mixture of intestinal and renal wastes, often became watery, but never was streaked with blood. Shortly before death the birds showed definite evidence of emaciation. The birds used to obtain the data in Table I were not disturbed following irradiation. To determine the sequence of events in the peripheral blood and marrow an additional 25 birds were irradiated with 2000 r 24 hours after red and white cell counts had been made. Every 2-3 days thereafter 2 birds were exsanguinated and blood studies carried out (Table II). Despite the great variation in the total counts of the various birds and the difficulty of the counting technic(6) because of the nucleated erythrocytes, it is clear that within 48 hours after irradiation the number of leucocytes in the peripheral blood had dropped precipitously and after a week these cells had almost disappeared. However, within 2 weeks after irradiation their number began to increase. The erythrocytes showed similar though much less marked changes in number (Table II). During this period the blood uric acid level of 9 birds 2 to 22 days after irradiation averaged 6.4 mg % in a range of 5.3 to 8.0 mg %. This is within normal limits for the parakeet.

Pathologic findings. In birds dying after irradiation there were usually few gross lesions. Hemorrhages in skin and intestinal tract, so often noted in a number of mammals(7) were not encountered. Frequently one or both lungs showed hemorrhagic areas of consolidation. Histologically these were usually associated with a more or less luxuriant growth of a fungus, identified on culture as *Aspergillus*. These were not postmortem growths for they were found in birds examined immediately after death. This fungus is a frequent cause of pulmonary disease in a variety of birds(8). Occasionally widespread bacterial colonization was found in many organs, in the lungs this was associated with yellow miliary lesions. The latter were not true abscesses because leukocytes were absent, but tissue necrosis and fibrin deposits surrounded dense masses of bacteria which on culture proved to be *Staphylococcus aureus*.

The most striking changes were found in the hematopoietic tissue. In the normal adult parakeet the thymus and bursa of Fabricius have involuted; lymph nodes are absent, as in

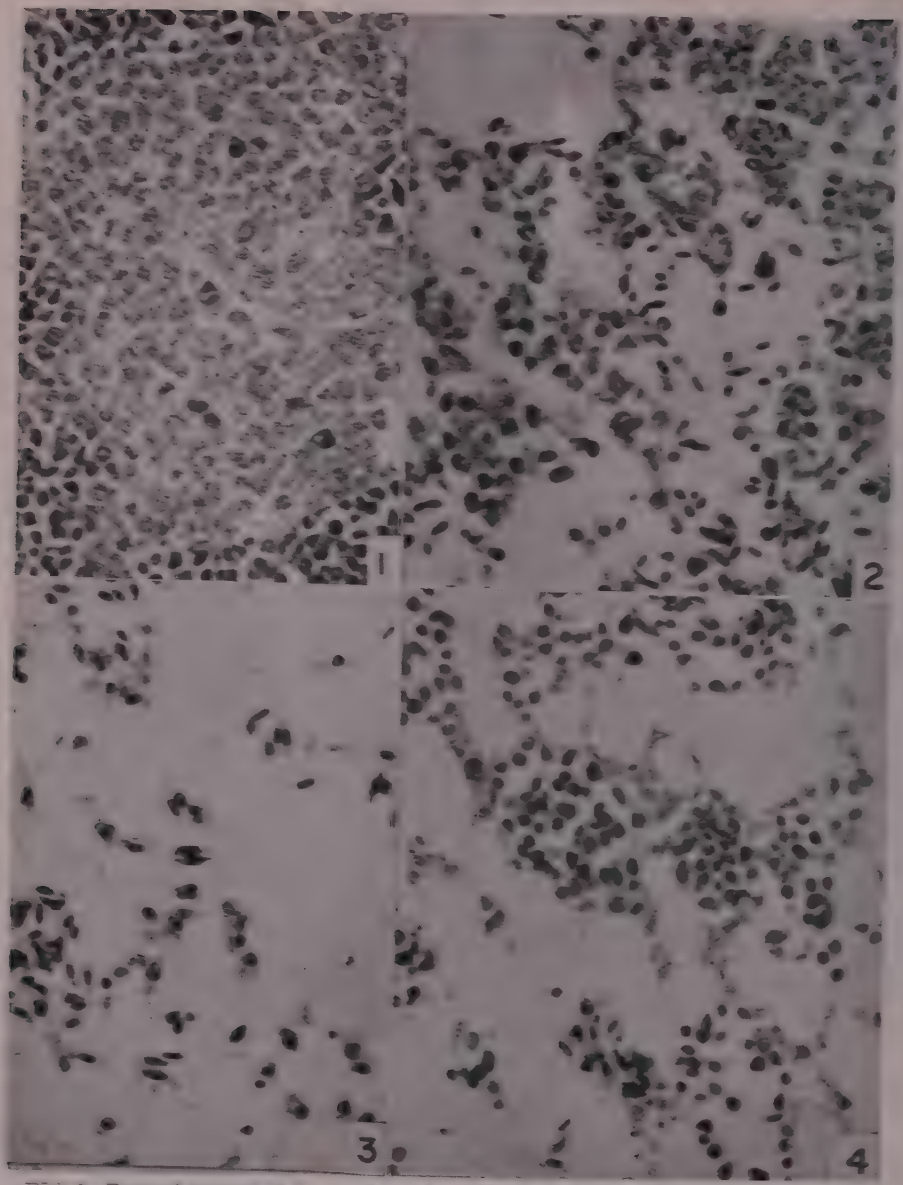

FIG. 1. Femoral marrow of a normal rabbit. Fat cells and clusters of granulocytic and erythrocytic cells are scattered between them. Many immature cells are present. Hematoxylin and eosin. Mag. × 430.

FIG. 2. Femoral marrow of an albino rabbit 4 days after exposure of head to 1000 r. Bone almost completely free of erythrocytes and granulocytes with large islands of primitive cells. Hematoxylin and eosin. Mag. × 430.

FIG. 3. Femoral marrow of an albino rabbit 4 days after exposure of head to 1000 r. A few mature nucleated erythrocytes are present in partially collapsed marrow. Hematoxylin and eosin. Mag. × 430.

FIG. 4. Femoral marrow of an albino rabbit 4 days after exposure of head to 1000 r. Marrow was selected to show an area of regeneration. Changes are beginning to be seen with promyelocytes and myelocytes. Hematoxylin and eosin. Mag. × 430.

most birds. The bone marrow of all parakeets dying 7-14 days after irradiation showed an almost complete absence of blood forming elements. The spleen was almost wholly depleted of lymphocytes. The sequence of changes was followed in the birds killed at intervals of 2-3 days after exposure to 2000 r.

In the normal parakeet marrow the erythropoietic cells occupy large blood sinusoids, granulopoiesis takes place in the interstitial tissue (Fig. 1). Within 48 hours of irradiation there was a striking depletion of immature elements (Fig. 2) and after 4 days the marrow was almost wholly aplastic (Fig. 3). However, small nests of cells resembling lymphocytes could still be found sparsely scattered through the marrow during the first week after irradiation. These cells resemble those identified by Jordan(9) as "lymphocytes" and which he believes enlarge and become hemocytoblasts that then differentiate into the various cell types. By the end of the second week these centers of regeneration are increasing in size and the primitive lymphocyte-like cells are assuming the appearance of precursors of granulocytes and erythrocytes (Fig. 4). During the third week after exposure the marrow becomes solidly filled with hematopoietic cells.

The changes in the spleen parallel those in the marrow and are characterized by a rapid loss of lymphocytes. By the end of the second week lymphocytes reappear accompanied by extensive areas of erythropoiesis. The precursors of these cells have not been identified with certainty although the reticulo-endothelium appears to play an important role.

Significant lesions were not observed in the crop, esophagus, gizzard, or intestine. The absence of ulceration in the crop and gizzard is particularly surprising because of the presence in these organs of seeds and sand respectively. The traumatic effect of these materials would be expected to produce extensive erosions if the mucosa had been damaged by radiation. In several instances, however, a pulmonary fungus infection, particularly of the right lung seemed to be an extension of a similar lesion in the crop. This suggests that the crop, into which fungi are frequently introduced with the seeds, is more susceptible to mycotic invasion after irradiation.

The liver and pancreas showed no evidence of radiation injury. The cytoplasm of the renal tubular epithelium was granular and the cells were swollen. These renal changes usually disappeared after the first week; however many of the birds that have survived for many months are now dying in renal failure. This would suggest that in some instances irradiation led to a progressive nephritis similar to that occasionally seen in patients treated with more than 2000 to 3000 r over the kidney region. These and other late manifestations of radiation injury in the parakeet are still under investigation.

In birds dying after irradiation with 1250 or more roentgen units, active spermatogenesis had ceased and in the ovaries some of the ova showed degenerative changes. However, because irradiation was administered without reference to the cyclic nature of spermatogenesis and oogenesis in these birds, no definitive conclusions can be drawn from these experiments.

The pituitary, thyroids, and adrenals were weighed on a Roller-Smith torsion balance. No consistent difference in the weight of the endocrines of the irradiated birds was found when compared with that of normal birds. Histological studies were likewise without evidence of radiation effect.

Discussion. The LD 50-30 of 1800 r for parakeets is 3 times as great as that obtained with the monkey in this laboratory using similar equipment and technic with a delivery rate of 23 r per minute(10,11). The dose is also 3 times greater than that reported for most mammals(1) and over twice that for the frog(12) and goldfish(13). Most studies on birds have been carried out with fowl chicks(4,5) and embryos(2). From data available it is difficult to determine the LD 50-30 for adult chickens when irradiated at a rate of 20 to 45 r per minute, but it appears to be approximately 600 to 800 r(3). The reason for the unusual resistance of the parakeet is not clear. The fact that in birds the circulating erythrocytes and thrombocytes are intact nucleated cells rather than the effete

erythrocytes and platelets found in mammals may be significant. The loss of leukocytes from the peripheral blood and marrow is approximately as rapid as that observed in mammals, indicating that the cellular mechanism for defense against infection is depressed. The relative resistance of the parakeet intestinal tract, however, may be an important factor in limiting access of pathogenic organisms to the blood stream and thus permit survival. Fluid and electrolyte loss through vomiting, diarrhea, and hemorrhage, often observed in mammals, is also not as great in the parakeet.

The observation of Stearner *et al.*(4) that chicks exposed to 1000 r at a rate of 43 r per minute died within 24 hours in acute renal failure is of interest in view of the cloudy swelling seen in the renal epithelium of the parakeet. Although in the parakeet these changes were not accompanied by an elevation of the blood uric acid level, there is evidence of progressive kidney damage followed by renal failure months after irradiation. Kidney damage following ionizing radiation has also been observed in mice(14).

Summary. The LD 50-30 for adult parakeets exposed to total body irradiation by x-rays at the rate of 23 r per minute is 1800 ± 75 r. The destruction and regeneration of the hematopoietic tissue is similar to that observed in mammals. The kidneys may be more susceptible and the intestinal tract less so than the same organs in mammals.

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Plasma Iron Studies in Normal Beagle Dogs.* (22447)

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Plasma iron values ranging from 50 μg% to 292 μg%(1) and 71 μg% to 285 μg%(2) have been observed in mongrel dogs. Plasma iron values were therefore studied in a highly bred stock of dogs maintained under relatively standard environmental conditions. Studies included comparisons among animals, weekly fluctuations in individual animals, differences between 24-hour fasting and non-fasting states, sex differences, and correlations with weight and age. This study is part of a larger study on the effects of radioisotopes in beagle dogs.

Methods. The 36 animals are a part of a colony of about 350 beagle dogs. They are

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housed in modern kennels with outdoor and indoor facilities where they are permitted a moderate amount of exercise. They are under the constant care of a full-time veterinarian and a full-time staff of 5 kennel personnel. During the experimental period there were no bacterial infections observed, and no parasites were found on periodic deworming procedures. Blood was drawn into oxalated test tubes and the plasma was analyzed for total iron by a modification of a previously described procedure(3). All glassware used was carefully cleansed with detergent, tap water, potassium dichromate sulfuric acid cleaning solution, and double-distilled water. Daily blanks showed an average contamination of 5.7 μg/tube (4.8 μg to 6.3 μg). The mean deviation between 2 aliquots of 30 samples was 1.04% $\pm$ 0.4 (2 S.E.). The average deviation between duplicate samples from 2 sites of venipuncture in 4 dogs was 3.8% $\pm$ 2.6 (S.E.). The recovery of a known amount of standard iron solution added to 9 plasma samples was 93.8% $\pm$ 1.6 (2 S.E.).

Results. A summary of the data on 34 dogs, comparing 24-hour fasting, non-fasting, male, female, and total groups is presented in Table I. With 3 exceptions, the same animals that were tested following a 24-hour fast were tested 1 to 2 weeks later under non-fasting conditions. One determination at 1- to 2-week intervals for a total of 2 determinations was done for both conditions. There is no apparent difference between 24-hour fasting and non-fasting dogs. The statistics were computed on the basis of total determinations for each condition, rather than on the basis of means for each animal as was done with male, female, and total groups. Since no differences were found between fasting and non-fasting states, all the values for each animal under both conditions were used in computing the values for male, female, and total groups. There were no differences between the group of 20 males and 14 females. For the total group of 34 animals a mean of 167 μg% $\pm$ 32.3 (S.D.) and range 89 μg% to 286 μg% were found.

Two additional dogs and one of the above 34 (2 male and 1 female) were studied weekly for 16, 15, and 10 weeks respectively (Table II). Each individual dog showed a wide range of values and varying means with large standard deviations.

TABLE I. Statistical Summary of Plasma Iron Values under Different Conditions.

Group	No. animals	No. determinations	Mean $\pm$ S.D., μg %	Range, μg %
24-hr fasting	30	52	163 $\pm$ 36.3	92-286
Non-fasting	33	58	171 $\pm$ 35.4	89-277
Male	20	67	166 $\pm$ 26.1	89-284
Female	14	43	169 $\pm$ 44.1	92-286
Total	34	110	167 $\pm$ 32.3	89-286

The plasma iron of 28 dogs (mean of 2 to 4 determinations) ranging from 7.0 kg to 19.0 kg showed a small positive correlation with weight ($r = 0.39$, 0.05 level $= 0.37$). The plasma iron of 36 dogs was correlated with age over a range of 1 to 4 years with no apparent correlation ($r = 0.11$, 0.1 level $= 0.28$).

Since there are only 4 determinations for each dog, the following statistical procedure was used to summarize for the whole population variations observed from week to week in a single animal. A mean and its absolute deviations were computed for each of the 34 dogs. The grand mean of the individual mean deviations summarizes individual variations for the whole population. Hereafter this grand mean will be termed average variation. Its value is 22 μg% with S.D. of 11 μg%.

In Fig. 1 a dog's mean is represented with a plot of the average variation and its normal distribution. Two standard deviations (22 μg%) of the average variation (22 μg%) will include 95% of the mean differences. The average variation will be added to and subtracted from the individual dog's mean to show the expected variation. We are con-

TABLE II. Statistical Summary of Weekly Plasma Iron of 3 Dogs.

Group, wk	No. animals	No. determinations	Mean $\pm$ S.D., μg %	Range, μg %
16	1	16	139 $\pm$ 38.3	82-214
15	1	15	199 $\pm$ 64.2	110-292
10	1	10	161 $\pm$ 48.3	101-226

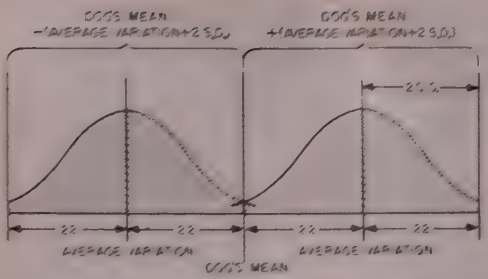

FIG. 1. Graphic description of variation of isolated plasma iron values in a dog *vs* its mean value.

cerned with the possibility of maximum deviation to be expected in a single animal; therefore, we consider the dog's mean + 22 μg% + 22 μg% (2 S.D.) and —22 μg% —22 μg% (2 S.D.). Simply stated, one would expect a control beagle dog's plasma iron over 2 to 4 determinations at 1- to 2-week intervals to fluctuate ± 44 μg% from its mean.

An analysis of variance to show the sampling errors of daily iron values out of the control population was made. Ten such days' samplings (8 samples per day) were analyzed with an F value of 4.5 which is beyond the allowed value of 2.67 for the 0.01 level. An unexplained difference of 2 days' values by the t test at the 0.05 level was found.

Discussion. In these dogs plasma iron showed no differences between 24-hour fasting and non-fasting states and no sex differences. There was no correlation between age and plasma iron over the range of 1 to 4 years. Over the weight range of 7.0 to 19.0 kg a low but definite positive correlation with plasma iron levels was observed. Using a standard breed of dogs in this study, a wide range of values similar to that in mongrel dogs was found. Since the weights of these animals remained constant over the testing period and the fluctuation was so variable, this variation cannot be attributed to weight changes.

Other investigators have found marked variations in people: 39 μg% to 170 μg% (4), 33 μg% to 221 μg% (5), and daily variations in a single person of the same magnitude as from person to person (6). If these large variations were due to fluctuating environment or genetic factors, they should have been reduced in this study. The plasma iron value fluctuates about 26% from a mean (167 ± 44) in a given animal of this study when the daily turnover from hemoglobin metabolism is about 300% of the total plasma content. The plasma iron concentration represents a very sensitive index to slight changes through the hemoglobin metabolism cycle of iron utilization and release.

A method was presented to characterize the normal variation in beagle dogs (Fig. 1). This statistical procedure will be used in following animals under various experimental conditions. One can then determine when a value falls outside the expected range for a given animal within 95% confidence limits and thus represents more likely a real change over the normal control situation.

Summary. Plasma iron in normal beagle dogs undergoes marked variation from week to week in a single animal, as well as from animal to animal. A low but significant positive correlation was found between plasma iron and weight over the range studied. No correlation was found with age under the conditions described. No differences were observed between 24-hour fasting, non-fasting, male, or female groups. A statistical method for characterizing weekly variation in a single animal has been presented. Reasons for this normal variation have been discussed.

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Relative Growth Responses of Previously Depleted Male and Female Rats to Vit. A Supplementation.* (22448)

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In quantitative estimation of vit. A by biological procedure, it has been considered necessary that tests be conducted in strict parallel with enough experiments to eliminate the effect of individual variability and with rigid control of all factors such as previous nutritional history, weight and sex of experimental animals, litter variability, and rate of growth during the test(1). It is generally believed that test animals from the same litter respond more uniformly than do rats from miscellaneous selection. To obtain reliable results, it is necessary that test animals from different litters be equally distributed among the assay groups. In assigning litter-mate rats to these groups, it is important that sex and body-weight (depleted) be evenly distributed among the groups. There have been differences of opinion, as to the influence of certain of the above factors on outcome of the assay. Sherman and Smith(2) stated, "There is clearly a tendency toward a reverse relationship between body weight of animal and its rate of gain upon a limited allowance of vit. A," " . . . that among animals of same species and age, the amount of vit. A required to support a given rate of growth tends to vary with size of the individual." However, the influence of sex of test animal on results of assay seems somewhat less definite. According to their findings, the difference in mean weekly growth-rate of 148 male rats compared to a like number of female rats over 8-week test, in which each animal received daily the same but limited quantity of vit. A was 0.3 g (total gain 24.6 and 22.3 g, respectively). As this difference appeared of questionable significance statistically, the authors suggest that, " . . . for investigations in which an extraordinary degree of refinement is sought, it might be slightly preferable to work entirely with animals of the same sex." Coward determined standard deviation of response of depleted male and female rats to doses of vit. A administered daily over a 5-week period(3). She found the standard deviation of increase in body weight of male rats to be greater than that of females and the curve of response of the male rat to a dose of vit. A to be greater than that of the female, under comparable experimental conditions. It was her opinion that, if test animals are properly depleted of their body reserve of vit. A, no greater accuracy is obtained through use of isogenic pairs of animals than rats from different litters(4). Other investigators have reported that under certain conditions male rats make greater gain in body weight while receiving a restricted intake of vit. A than do female rats under comparable conditions. Some authors have attributed the difference in growth rate to difference in body weight of the 2 sexes at beginning of the assay. Data obtained during the last few years in the course of vit. A-carotene assays and vit. A storage studies shed further light on the importance of the above mentioned precautions in carrying out biological assays for this vitamin and are presented herewith.

Methods and materials. Young rats ranging from 21 to 24 days of age and between 42 to 48 g in weight were placed in individual all-metal cages having raised screen floors. Each rat was given a liberal but known amount of U.S.P. vit. A-deficient diet(1) and distilled water. The caging, feeding weighing, and care during depletion period and subsequently during supplementary feeding period were carried out essentially as recommended in the U.S.P. technic. Each rat comprising an assay group received for 28 days the same amount of U.S.P. vit. A standard, or of crystalline vitamin or provitamin as a supplement

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TABLE I. Effect of Dosage of Vit. A on Relative Growth Response of Male and Female Rats Previously Depleted of Their Body Reserve of the Vitamin.

	Males				Females				
Vitamin fed daily*	No. of rats	Depletion wt, g	Gain in wt, g †	Gain/28 days wt, g	No. of rats	Depletion wt, g	Gain in wt, g †	Gain/28 days wt, g	Ratio of ♂ to ♀ gain
1.	13	113	26 ± 2.5	26	8	111	28 ± 2.9	28	.93
1.07	96	110	32 ± 2.7	30	78	109	34 ± 2.4	32	.91
1.4	22	117	40 ± 2.4	29	10	115	39 ± 3.1	28	1.03
1.5	210	115	46 ± 4.9	31	174	114	42 ± 5.0	28	1.09
1.93	30	119	62 ± 3.6	32	27	115	56 ± 4.2	28	1.17
2.0	71	114	67 ± 5.8	34	69	110	55 ± 4.6	27	1.22
2.9	19	113	79 ± 5.2	27	11	111	64 ± 3.8	22	1.23
5.7	8	121	86 ± 4.9	15	3	119	65 ± 3.1	12	1.32
10.	7	116	80 ± 6.9	8	4	113	62 ± 5.9	6	1.29
18.	8	107	80 ± 5.4	4	6	107	66 ± 3.6	4	1.21
25.	21	107	84 ± 4.3	3	6	102	62 ± 6.3	2	1.35
36.	8	107	92 ± 6.7	2	2	103	54 ± 6.7	2	1.52
40.	3	104	103 ± 3.2	3	3	101	70 ± 4.3	2	1.47
50.	23	102	83 ± 7.4	2	5	107	65 ± 5.1	1	1.28
70.	48	104	99 ± 6.2	1	24	105	71 ± 6.5	1	1.39

* U.S.P. units or estimated equivalent. † Mean wt with stand. error.

to the deficient diet. These supplements were dispensed in a purified cottonseed oil medium which was of such concentration as would limit the amount of solution fed to each rat to 0.7 ml per week. This was administered as 2/7 of the weekly allotment on Monday, 2/7 on Wednesday, and 3/7 on Friday. The calculated daily dosage of vitamin or provitamin ranged from 1.0 to 2.9 U.S.P. units in the assay studies and as high as 70 U.S.P. units/day during vitamin storage studies. During the 4-week test period a record was made of food consumption, body-weight changes, and general appearance of the animals. Data pertaining to mean depletion weight of several groups of animals and to mean growth response during the test period have been compiled and arranged according to sex and increasing daily dosage of the vitamin and are presented in Table I. In addition, typical data reflecting the relative weekly growth response of male and female rats to 4 different dosages of vitamin are presented in Fig. 1.

Results. Data in Table I reveal that there may be a measurable difference in growth response of previously depleted male and female rats to a supplementary allowance of vit. A. This difference was insignificant when daily dosage ranged 1.0 to 1.5 U.S.P. units or in the range generally employed in biological assay for this vitamin. There was some evidence that the female rat makes the greatest growth response to vit. A supplementation when the daily allowance of vitamin is 1 U.S.P. unit or less. When less than 1 unit was employed, it was usually difficult to evaluate, owing to wide variation in responses of test animals and to other complications. When daily dose of the vitamin ranged from 2 U.S.P. units upward, the male rats grew more than did the females. This difference in growth rate associated with sex was observed consistently and appeared to increase at a gradual rate until daily dosage of the vitamin reached 36 to 40 units. That this difference in growth rate was due to differences in body weights of male and female rats at the beginning of the test period seems untenable from the data at hand. The optimal rate of growth for male rats was 21 to 25 g per week and that for female rats 16 to 18 g. That this difference in growth is not due to differences in sexual development during the period of supplementary feeding is suggested by the data presented in Fig. 1. Here, it is to be noted that, while male rats receiving the higher rates of vitamin supplementation grew at a more rapid rate than did the corresponding females, there was no consistent evidence that either the male rats increased their rate of growth or that the female rats decreased their rate of growth during the 28-day test period. Food intake records indicate a direct relationship between food intake and growth

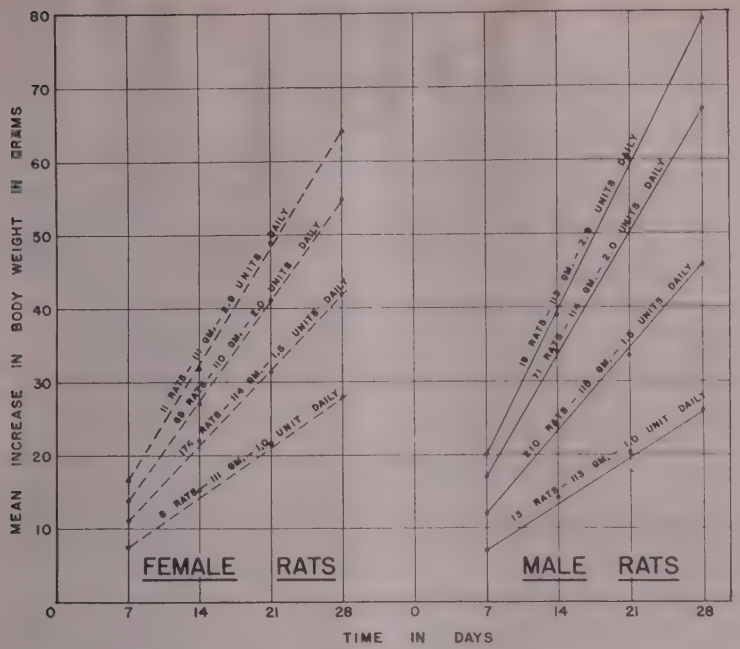

FIG. 1. Relative growth response of male and female rats to 4 different dosages of vit. A administered over a 28-day period.

in both sexes.

Another observation from data in Table I is the relatively similar growth response of both male and female rats to vit. A supplementation within dosage range of 1 to 2 U.S.P. units vit. A daily. This suggests that within this range growth response is a linear function of the vitamin dose. These data also suggest the precaution that should be exercised in interpreting data obtained while attempting to evaluate the relative effectiveness of beta carotene and vit. A in promoting growth in vit. A deficient rats when the carotene is fed at 1 μg (1.67 International units by definition) per day and vit. A at 1 μg (3.33 U.S.P. or International units) per day, or at any dosage range where either of these supplements produce a growth response out of proportion to the dosage.

Summary. Male rats which have been depleted of their body reserve of vit. A grow more during a 28-day test period than do corresponding female rats when a daily dosage of 2.0 U.S.P. units or more of the vitamin is fed as the supplement. This increased growth is not directly related to the body weight of the test animal at the beginning of the supplementary feeding period. While it appears that the greater growth capacity is an inherent characteristic of the male rat which is present at the beginning of the test period, it is not clear as to why this characteristic does not manifest itself at all levels of vit. A intake.

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Utilization of Glucose by Hyperthyroid Isolated Rat Liver.* (22449)

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The marked stimulation by thyroxine of the utilization of carbohydrates by peripheral tissues and liver has been demonstrated by McEachern(1). *In vitro*, such stimulated tissues have markedly increased oxygen consumption and increased rate of glycolysis. It seemed that the behavior of the isolated perfused liver of a hyperthyroid rat should provide information concerning glucose metabolism in a simplified system but one which retains anatomical integrity lost with the use of liver slices. The present studies were undertaken to determine the effect of thyroxine on utilization of glucose by the liver.

Materials and methods. Male rats (Long-Evans strain) 16-20 weeks of age were used. These animals were fed stock laboratory diet† to which 0.1% thyroxine was added, for two weeks prior to time of sacrifice. Livers from these hyperthyroid animals were removed and perfused as isolated surviving organs for 6 hours according to previously described technic(2,3).‡ The glucose content of the perfusate was adjusted to contain an initial level of 650 mg % of glucose. Our previous experience, and that of other investigators, indicated that glucose utilization by the isolated liver preparation could not be demonstrated except at high initial glucose concentrations (4), and that entry of glucose into cells is facilitated by high glucose concentration(5). As controls, livers from untreated animals, fed standard laboratory maintenance diet, were removed and perfused in identical manner. Cellular elements and plasma, incorporated into perfusion fluid, were obtained from normal, untreated rats in all experiments with normal and with hyperthyroid livers. There were 8 perfusions of isolated livers from the hyperthyroid group, and 8 from control group. Glucose level of perfusate was determined by Nelson's modification of the method of Somogyi(6).

Results. Group I—Control studies. The average initial glucose concentration of the perfusate in this group was 641 mg % (range: 520-710 mg %); at the end of 6 hours of perfusion the glucose level had fallen an average of 22% of initial value. Hourly changes in glucose levels are given in Table I. Considerable irregularity in glucose level was observed from hour to hour, and from experiment to experiment, when normal livers were used. Indeed, at times the glucose concentration in the perfusate increased. (Table I).

Group II—Hyperthyroid studies. The average initial glucose concentration of the perfusate was 636 mg % (range: 580-710 mg %); the glucose level fell to an average of 52% of initial values at the end of 6 hours perfusion. Average hourly changes in glucose levels are given in Table I. The fall in glucose concentration was fairly regular and steady throughout the 6 hour perfusion.

Discussion. When the liver of a rat, which has been on daily intake of thyroxine, is perfused, glucose disappears from the perfusate rapidly, compared to rate of disappearance during perfusion of liver from an untreated animal. The data in these experiments thus indicate that the rate of utilization of glucose by the isolated hyperthyroid liver is markedly accelerated, and, in this respect, confirm in perfusion experiments the *in vitro* tissue slice studies of McEachern.

Using normal livers, it is difficult to demonstrate glucose utilization by the isolated rat liver perfusion experiments except when high

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† Simonsen Animal Farms, Gilroy, Calif., Maintenance Diet No. 2 consisting of ground wheat, corn, linseed meal, milk powder, meat and fish meal, alfalfa meal, yeast, vitamins and multiple salts supplement.

‡ The bovine albumin used in the perfusate for these experiments was kindly furnished by Dr. Sanford L. Steelman of Armour and Co.

TABLE I. Glucose Concentration of Perfusion Fluid. Expressed as avg % loss from initial concentration.

Group I. Livers from untreated rats—8 exp.							
Perfusion time in hr	0	1	2	3	4	5	6
% fall in glucose conc.	0	11	14	24	28	20	22
S.E. of mean*	±	± 6.6	± 9.2	± 10.8	± 13.1	± 15.5	± 14.2
Group II. Livers from thyroxine fed rats—8 exp.							
Perfusion time in hr	0	1	2	3	4	5	6
% fall in glucose conc.	0	4.3	24	34	45	52	52
S.E. of mean	±	± 6.8	± 12.8	± 10.5	± 10.6	± 9.3	± 8.7
S.E. of diff. between means†				± 5.5	± 6	± 6.4	± 5.9

* S.E. of mean $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$. † S.E. of difference between means $= \sqrt{\frac{\theta_1^2}{N_1} + \frac{\theta_2^2}{N_2}}$.

initial glucose concentrations of perfusate are employed(7).

It may well be that synthesis of glucose or glycogenolysis is adequate in the normal liver to supply glucose to the perfusion fluid in sufficient amounts to overbalance utilization by the perfusion system. Irregularities in such supply and utilization would account for irregularities observed in concentration of glucose in the fluid perfusing the normal liver. Presumably livers from thyroxine-treated animals are unable to supply sufficient glucose to overcome the steady utilization at the relatively rapid rate observed in our experiments. In contrast, the hyperthyroid liver effects a rapid disappearance of glucose from the perfusate when the initial glucose concentration is either at high or at physiologic levels(7).

The fate of the glucose which disappeared from the perfusate during these experiments cannot be determined from the present data. Some of the glucose which disappears from the perfusate during perfusion in all likelihood is converted into glycogen, since hyperthyroid livers uniformly show an increase in glycogen content after six hours of perfusion (8).

Summary. Perfusion of the isolated hyperthyroid rat liver results in a marked fall in the perfusate glucose level due to accelerated utilization of glucose by the hyperthyroid liver tissue in comparison with that of the isolated liver from euthyroid rats.

The authors express their thanks to Dr. Meyer Friedman, Director of Harold Brunn Institute for his suggestions, and to Dr. Shirley St. George and Sara Winderman for chemical determinations.

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Ethylene-Glycol Extracts of Leptospirae in Complement-Fixation Tests. (22450)

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(Introduced by Gilbert Dalldorf.)

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Recognition of leptospirosis as worldwide public health problem has developed from recent studies(1-4). It has also been established that as many as 9 serotypes of pathogenic leptospirae occur in the United States (4-6). In search for diagnostic test that is easily performed and adapted to large-scale examinations, several workers have investigated complement-fixing antigens. The antigens considered have been crude whole-cell preparations grown in artificial media(7,8), formalinized infected chorioallantoic fluids of chick embryo(9), products of sonic vibration (10), weakly phenolized salt solution suspensions of microorganisms(11), and a soluble-specific antigen liberated into medium in which leptospirae are propagated(12).

Broad spectrum leptospirae antigens obtained by sonic vibration are reportedly sensitive and specific in acute leptospirosis and permit the use of fewer strains in diagnostic examinations(13). Since the wide scope of these antigens should offer an easier approach to detection of leptospiral antibodies of various serotypes, isolation of a similar fraction was attempted by means of ethylene-glycol extraction. Ethylene-glycol extracts derived as described below yielded complement-fixing antigens which were easily prepared and sensitive in the detection of leptospiral antibodies.

Material and methods. Antigens were prepared from type strains of leptospirae grown in modified Korthof medium at 30°C for 14 days. Serotypes included two strains each of *Leptospira icterohemorrhagiae, L. canicola,* and *L. pomona.** Leptospirae were killed by formalin (final 0.2% concentration) and collected by centrifugation at 10,000 rpm for 20 minutes at room temperature in Sorvall angle head centrifuge. The extraction method was similar to Morgan's(14). After washing twice with isotonic phosphate buffered sodium chloride solution (pH 7.2) cells were suspended, using a syringe and needle, in volume of ethylene glycol representing 2% of original culture fluid. The suspension was shaken with glass beads. Extracts recovered as supernates after centrifugation at 10,000 rpm for 1 hour were dialyzed first against running tap water and finally against phosphate buffered sodium chloride solution. The opalescent solution recovered was heated 15 minutes in boiling water bath. Cell residues were extracted a second time with one-half the volume first employed. After preliminary testing to determine potency, satisfactory extracts of single strains were combined. *Rabbit antiserum* was produced with each strain used in antigen production. Rabbits were immunized by intramuscular injection of killed culture in adjuvant emulsion(15). *Test procedure.* Complement-fixation test technic was that employed in this laboratory(16) using three 50% units of complement with fixation period of 4 hours at refrigerator temperature (3-6°C). Reactivity is expressed in Tables II and III as Titer equals $D(K_{8A}-1)$.

Results. Rabbit antiserum: Ethylene-glycol extracts of leptospirae fixed complement in the presence of rabbit antisera. The antigens were sufficiently potent to permit dilution and were neither lytic nor anticomplementary in the range of maximum reactivity with homologous serum. Inhibition of complement fixation was evident in zones of anti-

* *L. icterohemorrhagiae* 3228—Dr. P. K. Olitsky. Isolated from wild rat. 5309—Dr. Martha K. Ward. Wijnberg. *L. canicola* 39660—Dr. K. F. Meyer. Isolated from blood of icteric dog 1937 (*J. Am. Vet. Med. Assn.,* 1939, v95, 710). 5305—Dr. Martha K. Ward, Communicable Disease Center, Chamblee, Ga. *L. pomona* 5160—Col. R. H. Yager, Veterinary Division Army Medical Center, Washington, D. C. Australis C. 5310—M. K. Ward, Communicable Disease Center, Chamblee, Ga. L9 NIH.

TABLE I. Specificity of Ethylene-Glycol Extract Antigens of Leptospirae in Complement Fixation Tests. Reactivity expressed as percentage of homologous antiserum.

		Rabbit antiserum produced with strain:					
		L. icterohemorrhagiae		*L. canicola*		*L. pomona*	
Extract antigen of strain:		3228	5309	39660	5305	5160	5310
L. icterohemorrhagiae	3228	*100*	10	33	33	33	33
	5309	10	*100*	33	66	33	66
L. canicola	39660	10	10	*100*	100	33	33-66
	5305	10	10	100	*100*	33	33-66
L. pomona	5160	10	10	66	33-66	*100*	100
	5310	10	10	33	33-66	100	*100*
"Sonic vibrated leptospiral antigen"*							
L. pomona		10		33		*100*	

* Antigen kindly furnished by Col. C. A. Gleiser, Army Medical Service Graduate School.

gen and serum excess.

As shown in Table I antigens had pronounced serogroup specificity in tests with rabbit antiserum. In addition, type specificity within a serogroup was demonstrated with strains *L. icterohemorrhagiae* (3228) and *L. icterohemorrhagiae* Wijnberg (5309). The degree of cross reactivity between serogroups was generally less at point of antigen concentration optimum for homologous antiserum. Results of comparative tests with sonic vibrated antigen are included in Table I. Similar relationships were demonstrated in tests refrigerated 16-22 hours.

Human serum. In tests with sera from human cases of leptospirosis diagnosed serologically, extracts appeared less serogroup specific than with immune rabbit sera, although not so broadly genus specific as to assure detection of all leptospiral serotypes with individual strain antigen (Table II).

Triple antigen. To obtain an antigen for screening tests, extracts of each of the 3 serotypes employed were combined to form a pool. Potency of the triple antigen thus prepared was equal to or better than that of the individual components. In some cases reactivity was enhanced, the antigen being more sensitive (Table II). Broad valency was shown by reactions obtained with specimens containing serum antibody of unrepresented types (*L. autumnalis* and *L. grippotyphosa* by agglutination).

Tests with specimens from 202 presumably normal human adults yielded a reaction rate of 9%; however, 7% of these were of weak reactivity (titers under 10), with some unconfirmed on retest.

TABLE II. Reactivity of Ethylene-Glycol Extract Antigens of Leptospirae in Complement Fixation Tests with Serum from Human Cases of Leptospirosis.

Extract antigen of strain:	Complement-fixation test titers with serum from cases: 1	2	3	Killed culture antigen of strain:	Endpoint of agglutination in tests of serum from cases: 1	2	3
1. *L. icterohemorrhagiae*				*L. icterohemorrhagiae* AB	32	8192	4096
a 3228	10	40	90				
b 5309	10	340	110				
2. *L. canicola*				*L. canicola*	0	256	2048
a 39660	10	20	60				
b 5305	10	30	70				
3. *L. pomona*				*L. pomona*	512	32	0
a 5160	10	30	60				
b 5310	10	50	80				
Triple antigens:							
1. Pool of 1a, 2a, 3a	20	120	110				
2. Pool of 1b, 2b, 3b	10	380	140				

TABLE III. Serologic Examinations of Serial Specimens from Human Cases of Leptospirosis.

Case No.	Collection date	Complement-fixation with triple antigen (ethylene-glycol extracts)	Hemagglutination*	Agglutination with killed culture of strain:† *L. icterohemorrhagiae* AB	*L. canicola*	*L. pomona*
1	8/23/54	220	1280			
	9/10	170	1280			
	10/28	20	160	32	0	512
2	7/11/55	280	640	4096	0	0
	8/15	120	160	8192	256	32
	10/24	10–20	160	1024	0	0
3	9/22/55	110	320			
	30	110	160	4096	2048	0

Clinical data:

Case 1. Male, age 30. Hospital admission 8/12/54 with malaise, chills, and fever for 2 days. Temp. between 101° and 104.6°. Complained of heaviness of head, stiff neck, and severe low back pain. Employed in slaughter house eviscerating calves, sheep, and lambs. Denied contact with adult cattle or pigs.

Case 2. Male; butcher. Onset of illness 7/2/55. Admitted to hospital 7/8/55 with temp. of 103°, headache, nausea, vomiting, and muscle aches particularly in calves of legs. Jaundiced 3 days; several bouts of loose stools. Improved without antibiotics. 7/19/55 WBC 26,000; CSF: elevated protein and cells. Drank Genesee River water in mid May 1955. Cut finger on meat cutting job June 1955. Contact with pet dog ill in June.

Case 3. Male, age 21. Admitted to hospital 8/31/55 with 2-day history of abdominal pain, nausea, vomiting, chills, fever and headache. Temp. 105°, muddy sclera, and abdominal tenderness. Jaundice 5th hospital day. Patient swam in canal in Tonawanda 10 days before admission.

* Chang's technic(19). Reactivity is expressed as highest serum dilution exhibiting definite agglutination (2+ or greater).

† Tests kindly performed at Leptospira Research Laboratory, Communicable Disease Center, Chamblee, Ga., Mrs. Mildren Galton in charge. Agglutination reported with certain of other serotypes employed was less than maximum recorded here. All dilutions started in range 1:16-1:160.

Three human cases of leptospirosis were brought to our attention during this study. Some of the serial specimens were insufficient for complete serologic examination. With at least one specimen from each patient, however, confirmatory data were furnished by agglutination tests with strains of numerous serotypes of leptospirae.† Results of serologic tests, agglutination, complement-fixation (with triple antigen), and hemagglutination, are recorded in Table III.

Our ethylene-glycol extract antigens are now routinely employed in serologic examinations for evidence of leptospirosis and have yielded reproducible results. They have retained reactivity when stored at –20°C or at 3-6°C for at least 3 months.

Composition of ethylene-glycol extracts of leptospirae was not studied. Proteins are said to be relatively insoluble and bacterial polysaccharides readily soluble in this reagent. Morgan and Partridge(14,17) identified products obtained by similar extractions of *Shigella dysenteriae* with diethylene glycol as loosely bound complexes consisting of 3 principal components: a carbohydrate which behaved as a hapten and was responsible for serologic specificity; a phosphorous containing conjugated protein which was antigenic in itself; and a nonantigenic phospholipid. The antigen described yielded a positive Molisch test and a negative biuret test.

Discussion. In view of the incidence of reacting sera in the normal population, examination of serial specimens, before making a diagnosis, is the routine practice of many workers regardless of serologic tests routinely employed.

In agglutination tests of specimens from patients with leptospirosis, antibody concentration increases from the first (acute-phase)

† Kindly performed at Leptospira Research Laboratory of Communicable Disease Center in Chamblee, Ga., under direction of Mrs. Mildred Galton.

to the second (convalescent) serum specimen. On the other hand, in complement-fixation tests with ethylene-glycol extracts of leptospirae, titers reached a maximum early in the disease. Thus a decline in complement-fixing titer from the first to the second specimen would not necessarily obviate a diagnosis of leptospirosis.

Extracts of leptospirae made with desoxycholate solution in a manner similar to that reported for the preparation of *Treponema pallidum* complement-fixation test antigen by Portnoy and Magnuson(18) proved reactive with rabbit antiserum but poorly reactive with serum from human leptospirosis.

Summary. A colloidal aqueous solution obtained by ethylene-glycol extraction of leptospirae appears to offer a complement-fixing antigen of considerable promise. Extract pools have broad valency and high sensitivity in the detection of leptospiral antibodies.

We wish to acknowledge the technical assistance of June McVeigh and Catherine Moczulski.

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Mechanism of Oxygen Deficit.* (22451)

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After studying the origin of oxygen debt during human exercise, Hill, Long, and Lupton(6) concluded that it resulted entirely from accumulation of lactic acid. Margaria and his co-workers(10) divided the oxygen debt repayment curve into two components, a rapid one with no significant decreases in lactic acid, and a slower one during which accumulated lactic acid was removed. These workers observed no accumulation of lactic acid during exercise producing oxygen debts of less than 2.5 l in man. They postulated that the component of the debt which is rapidly repaid results from a depletion of phosphocreatine in the muscles. A number of observations seem to support this theory. Lundsgard(9) showed that in anaerobic conditions, lactic acid accumulates and organic phosphate dissipates in muscle in tetanus, and that phosphorylation follows a short tetanus. Tiegs(15) found much more creatine, a fission product of phosphocreatine, in fatigued than in rested muscle, a finding confirmed by Eggleton(2) who observed three times the

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normal amount of creatine in fatigued muscle. When Tiegs placed fatigued muscle in an oxygen stream, the creatine level decreased. Grollman(3) reported a decrease in phosphocreatine in rat muscle during exercise and noted interesting correlations between phosphocreatine levels and breakdown in conditioning. Hansen(4) also found that a debt incurred at low exercise levels apparently is not accompanied by accumulation of lactic acid. In discussing the mechanism of this debt, he showed that it was not due to either increase in arteriovenous oxygen difference during exercise or circulatory deficiency. He also concluded that a drain on oxygen stored in the muscle and bound to myoglobin is not the responsible factor even though an oxygen debt of 1-5 cc per 100 g of muscle would occur if myoglobin were saturated at rest and completely unsaturated during exercise. Hansen felt that myoglobin serves as an oxygen supply during contraction when circulation is shut off, and is resaturated between each contraction. The myoglobin saturation curve lies at quite low oxygen tensions; that whatever the role played by myoglobin, it does not bind sufficient oxygen to account for the 2.5 l debt that can occur without lactate accumulation. Belitzer(1) found that adding creatine to pulped muscle increased its oxygen consumption while adding phosphocreatine had no effect. He concluded that respiration is increased by addition of a phosphate acceptor. Niemeyer, Crane, Kennedy and Lipmann(11) observed that respiration of liver mitochondria is increased up to 50% by addition of a phosphate acceptor system, namely hexokinase plus glucose. This removes phosphate from ATP, forming ADP which is a phosphate acceptor, and ATP is regenerated by oxidative processes. Controls with glucose-6-phosphate, hexokinase, or glucose alone were negative. It appears, then, that with muscular exercise, a considerable oxygen debt is incurred which is not explained by accumulation of lactic acid. This debt may result from depletion of high-energy phosphate in muscle during exercise and an increase in oxygen consumption caused by an increase in phosphate acceptor. In this complex equilibrium there may be a mass action effect—rate of oxidation, and thus of oxygen consumption, being regulated by rate of high-energy phosphate depletion and phosphate acceptor accumulation in the muscle. Henry (5) suggested that oxygen consumption during exercise cannot increase until there is a substrate to be oxidized, and that substrate is formed as a result of work. Thus, time would be required for accumulation of a substrate which would be responsible for the debt. If the word 'substrate' were replaced with the words 'phosphate acceptor' and the word 'oxidized' placed in quotes, his idea would resemble the hypothesis we are proposing. The systems would differ markedly if there were uncoupling of phosphorylation. This concept is consistent with the hypothesis that energy for contraction is stored in muscle structure, released during contraction, and restored from chemical stores during relaxation(13).

In this study, the debt calculated from change in lactic acid and inorganic phosphate concentration in the isolated canine gastrocnemius muscle was compared with the oxygen deficit calculated from oxygen consumption.

Methods. The preparation used was similar to that of Kramer and Quensel(7). A long incision extending from the femoral triangle to the tendo calcaneus was made on the medial side of the hind limb. The long saphenus artery, vein, and nerve were cut between two ligatures and reflected from the medial aspect of the knee. The sartorius, gracilis, semitendinous, and semimembranosus muscles were separated, at their insertions, from the tibia and the gastrocnemius muscle. The femoral vein, the short saphenus vein, and the posterior femoral vein were cleaned. The short saphenus and posterior femoral veins were ligated below their last branch from the gastrocnemius muscle. All branches not from the gastrocnemius muscle were ligated and cannulae for collecting and returning venous blood were inserted into the femoral vein. The tibial nerve was cleaned, ligated, cut, and placed upon stimulating electrodes. Blood flow was determined at 5- or

15-second intervals with a direct volume collector recording via a spirometer on a kymograph. Oxygen saturation in venous blood was recorded continuously with a Kramer arterial oximeter. In each experiment, the apparatus was calibrated, and arterial saturation was determined by Roughton and Scholander's method(14). The muscle was stimulated for 1-second intervals with 1 second rest at 300 cycles per second (See 7 and 12). After an initial run, the muscle was rapidly excised during stimulation and placed in liquid air. The contralateral gastrocnemius was immediately removed as a control. Lactic acid was determined by the method of Barker and Sommerson(16), true inorganic phosphate by the method of Lowry and Lopez(8), and total phosphate (7-min. phosphate) by the method of Fiske and Subbarow(16). Oxygen deficit was calculated from the chemical determinations of high energy phosphate and lactate. Assume: 34 mol~P formed per mol glucose oxidized per 6 mol O_2 used $\therefore$ 0.1277 cc O_2/mg P. Assume from glucose 1 mol~P per mol lactate. Each mol lactate formed is equivalent to 1 mol of~P reconstituted. 1 mol~P equivalent

FIG. 1. Changes in oxygen uptake, blood flow and venous oxygen content with time in gastrocnemius muscle. Stimulation of cut tibial nerve indicated between 2 and 7 min. Blood sample taken during final minutes of exercise. During similar experiment the gastrocnemius was excised at end of 2 min. stimulation.

TABLE I.

	Date	Lactate, mg/100 g			~P, mg/100 g			O_2 deficient, cc/O_2/100 g		Blood O_2			O_2 cons/100 g & muscle, cc/min.		
		Rest	Stim	Δ	Rest	Stim	Δ	Chem	Blood	Ven. rest	Ven. stim	Arterial	Ex.A-V diff.	Rest	Exercise	Δ
1.	7/20	27.2	54.9	27.7	85.1	65.1	20.0	1.88	—	14.2	3.1	17.4	14.3			
2.	25	—	—	—	—	—	—	—	4.13	15.5	5.5	22.3	16.8	1.73	13.50	11.77
3.	27	9.8	21.9	12.1	55.4	41.6	13.8	2.29	2.94	10.8	5.0	15.5	10.5	.89	6.67	5.78
4.	8/ 1	20.7	56.5	35.8	84.5	57.8	26.7	4.98	1.59	15.8	2.5	18.4	15.9	.68	9.75	9.07
5.	3	8.1	14.3	6.2	80.9	67.5	13.4	1.98	2.80	13.2	5.2	18.2	13.0	.80	5.15	4.35
6.	9	13.3	35.6	22.3	—	—	—	—	3.14	14.0	6.4	20.0	13.6	.98	8.58	7.60
7.	11	21.9	32.0	10.1	—	—	—	—	2.69	17.2	7.5	19.2	11.7	.23	4.19	3.96
8.	16	10.7	19.2	8.5	67.9	55.0	12.9	2.01	—	11.0	4.0	16.5	12.5	.38	3.40	3.02
9.	18	8.6	11.0	2.4	61.4	53.4	8.0	1.13	.91	14.0	4.6	19.2	14.6	.80	3.49	2.69
10.	19	20.0	29.9	9.9	65.3	53.3	12.0	1.97	1.03	15.0	6.0	18.8	12.8	1.35	7.53	6.18
11.	22	—	—	—	—	—	—	—	.78 per muscle	16.2	8.8	18.1	9.3	—	—	—
12.	23	8.8	19.2	10.4	71.6	52.9	18.7	2.84	1.26	11.8	4.7	16.3	11.6	1.11	8.36	7.25
13.	29*	12.3	21.6	9.3	67.0	62.7	4.3	0.91	1.60	13.7	4.0	18.1	14.1	2.40	10.68	8.28
14.	31*	9.5	33.8	24.3	63.6	50.4	13.2	2.76	2.10	12.5	5.0	14.1	9.1	1.31	11.60	10.29
Avg		14.2	29.2	14.9	70.3	56.0	14.3	2.28	2.20	13.9	5.2	18.0	12.8	1.06	7.74	6.68
Surgical control†		9.1	7.9	1.2	62.5	62.4	0.1	.04 on control (rt.) leg								

* Muscle taken after 45 sec stimulation; all others after 2 min. † Muscle taken without stimulation.

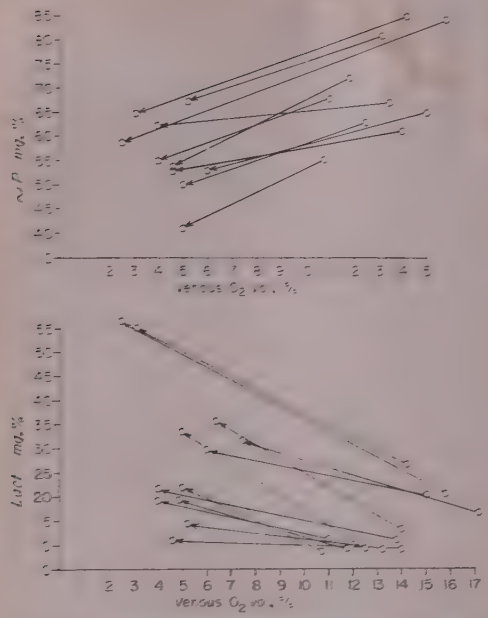

FIG. 2. Changes in organic phosphate (upper) and lactic acid (lower) with exercise plotted *vs* venous oxygen content. Individual points at rest and exercise are connected by arrows indicating shift with exercise.

to 3.96 LO_2, 1 mol lactate formed is equivalent to 3.96 LO_2, ∴ .044 cc O_2 deficit per mg lactate formed. The actual oxygen deficit was obtained from the oxygen consumption data by determining the difference between the oxygen consumption during the initial period and the steady state oxygen consumption.

Results. A typical experiment is shown in Fig. 1, and the data from all experiments appear in Table I.

In every case, the high energy phosphate decreased during exercise, and the muscle lactate increased. These differences are significant at the 0.1% level. The relationship to venous oxygen tension is shown in Fig. 2.

Assumption that oxygen debt is dependent on the change in the level of high-energy phosphate and calculation of "lactate debt" on the basis of high-energy phosphate replacement would indicate a relationship between depletion of high-energy phosphate and the increase in lactate during exercise. Fig. 3 illustrates this relationship in these experi-

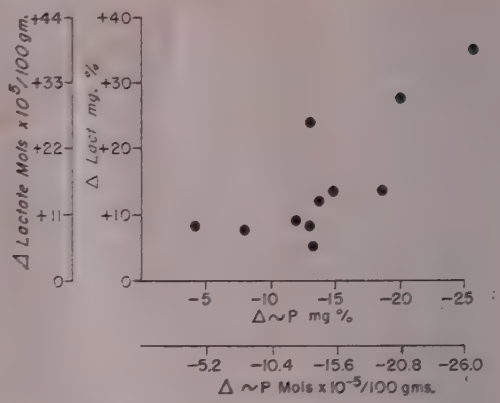

FIG. 3. Change in lactic acid with exercise *vs* change in organic phosphate.

ments. Although the absolute levels of oxygen consumption are not directly related to the level of phosphate acceptor, the ratios of change are proportional (Fig. 4).

The calculated oxygen deficit (Table I) agrees well with the measured deficit.

Discussion. The results of the experiments are compatible with the assumption that oxygen deficit is related to depletion of high-energy phosphate (increase in phosphate acceptor) and accumulation of lactic acid. Clearly, the role of myoglobin is small.

It is of interest to note that lactic acid appeared in the muscle during these experi-

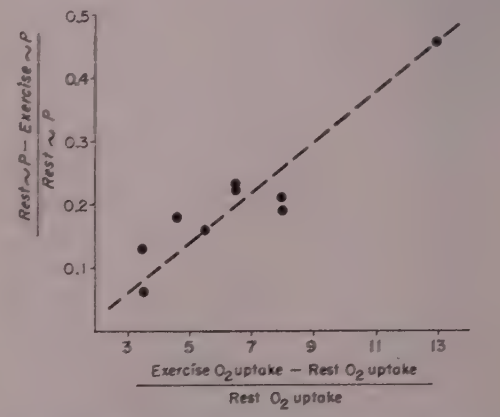

FIG. 4. Relation of change in phosphate acceptor to oxygen consumption expressed as ratio of change in organic phosphate to resting level of phosphate on ordinate, and ratio of increase in oxygen consumption to resting oxygen consumption on abscissa.

ments. The oxygen consumption of exercise (7.7 cc/100 g muscle) is not large (about 1 l/min in a man with 14 kg of muscle active). From these experiments it seems reasonable to suggest that lactic acid is formed in the muscle at any level of work, and that the lactate relationship to oxygen debt observed by Margaria *et al.*(10) indicates a threshold mechanism in the whole-body metabolism of lactate. The direct relationship between the change in high-energy phosphate and the amount of lactate produced, as well as the relationship of the amounts to the work done, is evident in Lundsgard's experiments(9).

Further, it is assumed that lactate is produced to provide high-energy phosphate and the oxygen deficit is calculated on a high energy phosphate equivalent, then the final oxidation of lactate (1/5 oxidized to reconvert 4/5) would involve a greater oxygen uptake, and the oxygen debt would exceed the oxygen deficit, *i.e.*, in terms of high energy phosphate formed 1 mol lactate formed is equivalent to 3.96 LO_2. 1 mol lactate requires 67.2 LO_2 for oxidation. If 1/5 oxidized to reconvert 4/5, then debt for 1 mol of lactate is 13.4 LO_2 or $\frac{13.4}{3.96}$; 3 times the calculated oxygen deficit.

During the time of stimulation before sacrifice, some lactic acid undoubtedly was carried away in the blood. Assuming a change in the concentration in the blood equal to that in muscle (14.9 mg/100 g) the error would be of the order of 5%.

The blood flows with exercise are similar to those reported by Kramer and Quensel(7) and illustrate the rapid increase in blood flow through the muscle with exercise concurrent with the widening A-V difference. The return of the blood flow and the A-V difference to resting levels follows a prolonged but related time course. This would seem to emphasize the role of the muscle pump in exercise blood flow and separate local effects on blood vessels from the central nervous control.

Summary. In muscular exercise, a considerable oxygen deficit is incurred which cannot be explained by the accumulation of lactic acid. The hypothesis that this deficit is due to depletion of high energy phosphate in muscle and that the oxygen consumption is increased by an increase in phosphate acceptor was tested in the perfused dog gastrocnemius. Oxygen deficit was calculated from change in lactic acid and high energy phosphate after 2 minutes contraction. Oxygen deficit was calculated from oxygen consumption determined at 5-second intervals by measurement of blood flow and arteriovenous difference. For exercise, the muscle was stimulated at 300 cycles/second for 1-second intervals with 1 second rest. In 14 experiments, the average values of lactic acid were 14.2 mg % at rest, and 29.2 mg % during stimulation. The calculated deficit was 2.3 cc O_2/100 g muscle, and the measured deficit was 2.2 cc O_2/100 g muscle.

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Correlation Between Chemical Constitution and Capillary Activity of Adrenalocortical Hormones.* (22452)

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It has been shown recently that a close relationship exists between the resistance of capillaries (as measured by negative pressure method) and certain steroids of the adrenal cortex. Cortisone increased capillary resistance of human beings(1) and in experimental animals(2) whereas findings with desoxycorticosterone were contradictory(2,3). In the present paper experiments are described in which a large number of steroid hormones, both naturally occurring and artificially produced, were systematically investigated for their potential action on capillary resistance and for possible correlation between chemical structure and capillary activity. Some importance is lent to such correlation by the fact that the anti-inflammatory (anti-rheumatic) effect of steroids was found to be dependent upon particular features in chemical structure(4) and by the possibility that anti-inflammatory effect and capillary activity may be closely related properties.

Method and procedure. Twenty-four mature female adrenalectomized albino rats of Sprague-Dawley strain weighing 220-300 g were used. Total adrenalectomy was performed at least 2 months prior to the experiments. The animals were fed Purina Checkers enriched with raw horse meat. One percent NaCl served for drinking water. Great care and much time was given to train these animals. They were not used until they became real pets and were perfectly accustomed to lying on their backs in the palm of the investigator with virtually no restraint, so that the testing procedure did not afford any emotional reaction. This treatment is indispensable because untrained animals are likely to develop changes in capillary resistance (emotional stress response), which makes testing and proper evaluation of results difficult if not impossible(5). Even though protracted changes in capillary resistance (late capillary stress response) do not occur after adrenalectomy, fluctuations of a few hours duration (immediate capillary stress response) may be frequently encountered(6). In addition, untrained adrenalectomized animals often show the phenomenon of emotional capillary spasm which may easily lead to erroneous readings (5). Capillary resistance was tested on the abdominal skin with the negative pressure method. The apparatus and details of procedure were described in previous papers(2, 7). The least negative pressure applied for 60 seconds and capable of eliciting one or more petechiae was considered the value for capillary resistance. In this manner a control base line of daily values was established, whereupon animals received, in the musculature of the thigh, daily injections of steroid to be tested for 3 days, after which period capillary resistance was again determined. The same animals were used for testing several substances, but were given at least 2 weeks of respite between test periods. Each substance was tested at least in 2 animals. The average daily dose was 1 mg/100 g body weight in a fine physiological saline suspension prepared by trituration and shaking. Volume of injected material varied from 0.1 - 0.3 ml. If no change in capillary resistance was noted, daily doses were increased by 50%, and in case of no response, by 100%. If test remained negative even with this final dose, the compound was considered ineffective. When a compound, in 1 mg 100 g dose, caused a marked change in capillary resistance, the tests were repeated with diminishing doses until the least effective dose was determined. Thirty-six different steroids were studied in this manner.

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TABLE I. Capillary-Active Steroids.

	Least effective dose, mg/100 g body wt
Cortisone acetate	.250
Hydrocortisone acetate	.070
Δ^1-cortisone	.020
Δ^1-hydrocortisone	.050
Fluorohydrocortisone acetate	.025

Results. Control values of capillary resistance of test rats were found to fluctuate between 4 and 12 cm Hg, mean 7.5 cm. Five of the 36 steroids increased capillary resistance by 50-60 cm Hg when injected in initial dose of 1 mg/100 g body weight for 3 consecutive days. These effective steroids are: cortisone acetate (11-dehydro-17-hydroxycorticosterone acetate), hydrocortisone acetate (17-hydroxycorticosterone), Δ^1-cortisone, Δ^1-hydrocortisone and fluorohydrocortisone acetate (9-α-fluoro-17-hydroxycorticosterone acetate). When tests were repeated with gradually decreasing doses the following results were obtained (Table I).

Comparing the least doses which proved effective we may state that hydrocortisone was more potent than cortisone, whereas Δ^1-cortisone, Δ^1-hydrocortisone and fluorohydrocortisone were more potent than hydrocortisone.

Thirty-one steroids (Table II), of the 36 investigated, were ineffective when injected in initial dose of 1 mg/100 g, as well as when

TABLE II. Capillary-Inactive Steroids.

Steroid compounds	Ketone group at C_3	Double bond between C_4 & C_5	Ketone group at C_{20}	Hydroxyl group at C_{21}	Stable orientation at C_{17}	Ketone or hydroxyl group at C_{11}	α-Hydroxyl group at C_{17}
Corticosterone (Compound B)							x
11-Dehydrocorticosterone							x
11-Desoxy-17-hydroxycorticosterone						x	
Dihydrocortisone acetate		x					
17-Hydroxyprogesterone						x	
21-Desoxycortisone				x			
4-Pregnentriolone diacetate						x	
Desoxycorticosterone acetate						x	x
Pregnane-3α,17α,21-triol-11,20-dione-21-acetate	x	x					
Pregnane-17α,21-diol-3,20-dione-21-acetate	x	x					
21-Acetoxyallopregnene-3,20-dione		x				x	x
Progesterone				x		x	x
17-Hydroxypregnenolone	x	x		x			
21-Acetoxypregnenolone acetate	x	x				x	x
Androstenedione			x	x		x	x
Allopregnane-3,20-dione		x		x		x	x
Δ^4-Transandrostene-3,17-diol	x		x	x		x	
16,17-Oxydopregnenolone acetate	x	x		x		x	x
Δ^5-Pregnene-3β,20β-diol-3,20-diacetate	x	x	x	x			x
Δ^5-Pregnene-3β,20α-diol-3,20-diacetate	x	x	x	x			x
$\Delta^{5,16}$-Pregnadiene-3β-ol-20-one	x	x		x		x	x
α-Estradiol	x	x	x	x			x
16,17-Epoxypregnenolone acetate	x	x		x		x	x
Equilenine	x	x	x	x			x
16-Dehydropregnenolone	x	x		x		x	x
Etiocholane-3α,11β-diol-17-one	x	x	x	x		x	x
Estriol	x	x	x	x		x	x
Estrone	x	x	x	x		x	x
Androsterone	x	x	x	x		x	x
Dehydroisoandrosterone acetate	x	x	x	x		x	x

x indicates absence of structural features essential for capillary activity.

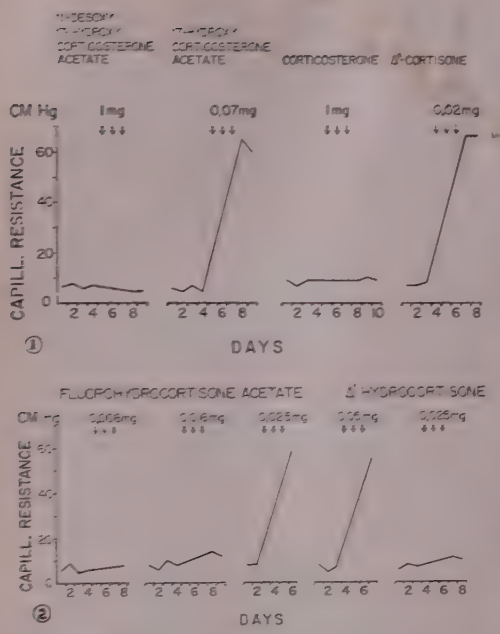


FIG. 1 and 2. Representative experiments showing tests for capillary activity of various corticoids. Individual tests are separated by at least 2 wk intervals. Arrows indicate intramuscular injections of the compounds, doses are given/100 g body wt.

dose was increased by 50% and later by 100%. Two figures serve for visualization of the testing procedure.

Comment. The chemical structure of the *5 steroids found to be "capillary active"* (ability to increase capillary resistance) has the following features in common: a ketone group at C_3; a double bond between C_4 and C_5; a ketone group at C_{20}; a hydroxyl group at C_{21}; a ketone or hydroxyl group at C_{11}; an α-hydroxyl group at C_{17}; stable orientation of the side chain at C_{17}.

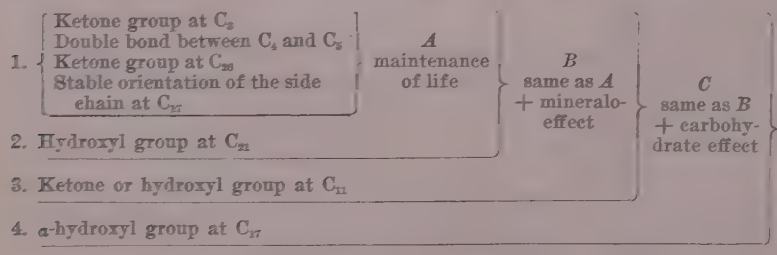
The *31 steroids found to be capillary inactive* are similar in one respect: at least one of the above 7 prerequisites is missing in their chemical structure (Table II).

To evaluate these findings, it is necessary to compare the already known relations between chemical constitution and biologic activity of corticosteroids. This relationship may be visualized in scheme shown at bottom of this page.

Items in 1 are necessary for maintenance of life. Items 1 and 2 are required for mineralo-effect beside maintenance of life. When items 1, 2 and 3 are present in the molecule there is an additional carbohydrate effect. For anti-inflammatory activity all items, 1, 2, 3 and 4 must be present in the molecule.

A comparison of chemical structural prerequisites, found in this study to be essential for capillary activity with those required for other types of cortical activity, indicates that they are identical with the prerequisites of the *anti-inflammatory effect* (*D* in the scheme). Our findings therefore suggest that the *capillary activity of corticoids, as measured by capillary resistance test, and their anti-inflammatory activity are closely related physiological properties.* The same relation is borne out by the comparative study of the 5 capillary-active steroids as to their least effective dose. We found that any alteration in the cortisone molecule which improves the anti-inflammatory activity also augments the capillary activity as measured by an increase in capillary resistance.

The present study suggests that capillary resistance test on the adrenalectomized rat may find a new practical application. From the clinical point of view the anti-inflammatory activity is one of the most important properties of a corticosteroid. Several meth-



ods have been devised to demonstrate and measure this activity. The described procedure is proposed as a new, simple, and convenient method for screening new compounds, either artificially prepared or later to be isolated from the amorphous fraction of the adrenocortical extract.

Summary. 1. To be able to increase capillary resistance a steroid must possess the same chemical structural prerequisites as required for anti-inflammatory properties. 2. Alterations in cortisone molecule which potentiate its anti-inflammatory activity also increase capillary activity. 3. It seems that capillary activity, as measured by the capillary resistance test, is intimately related with the mechanism involved in the anti-inflammatory effect. 4. Capillary resistance test on adrenalectomized rat is proposed as a new bio-assay method for the demonstration of the anti-inflammatory activity of corticoids.

For compounds investigated we are indebted to the research departments of the following firms: Ciba Pharmaceutical Products, Merck and Co., Parke, Davis and Co., Sharp and Dohme, Schering Corp., and E. R. Squibb and Sons.

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Effects of Lysergic Acid Diethylamide upon Performance of Trained Rats. (22453)

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Although lysergic acid diethylamide (LSD) is known to produce symptoms in man resembling those of certain psychotic disorders, a quantitative measure of the effects of this drug in animals has been lacking. Woolley(1) has described a syndrome observed in mice injected with LSD, and Shore *et al.*(2,3) have shown that LSD antagonizes the potentiating effects of reserpine and serotonin upon hypnotic agents. In the present paper, we demonstrate that the effects of LSD upon trained rats bear a linear relationship to the log dose of the drug, and that this phenomenon can be used in the study of LSD-antagonists.

Methods. Holtzman male rats of 100 to 225 g were used. Preliminary evidence indicates that animals outside this range may not be suitable, but this has not been established with certainty. Animals, fasted for 24 hours or longer, were trained to climb a vertical rope of about 172 cm as described previously(4). Incentive for climbing was food cup placed on platform at top of the rope; in addition, there was an electrified wire grid at the bottom. Also at bottom of rope was a small "tweeter" type of loud speaker which emitted brief bursts of noise at intervals of about a half-second. Untreated animals needed neither grid nor noise, but these facilitated climbing in rats affected by drugs, and decreased variability of performance. Observations were made of behavior of animals while in their cages as well as while negotiating the climb. Time required for the climb was obtained to the nearest 0.1 second. Three control times were obtained before administration of drug, and the average value was used as base line for drug effect, after it was established that in saline-injected animals climbing time remained constant for 5 hours. Evaluation of effects of drugs on climbing per-

formance was made as follows: first, the action of LSD was very rapid after intraperitoneal injection; it was quite marked within 5 minutes, usually maximal in 10 minutes, and after 20 minutes declined rapidly for about 10 minutes, then more slowly until original base line was approached within 1 to 2 hours after injection. Drugs which modified the effects of LSD were found to affect both magnitude and duration of prolongation of climbing time produced by LSD; also, both parameters were affected by dose of LSD. Therefore, determinations of climbing time were made at 5, 10, 20, 30, 45, and 60 minutes after LSD, and thereafter at half-hour intervals until recovery had occurred. Climbing times found in this manner, when plotted against time, produced points which could be connected by straight lines to form a polygon whose area could readily be calculated. The effects of drugs could be evaluated by their effects on area of this polygon. Climbing time of well-trained rats was usually 4 to 5 seconds. At peak of drug effect, this would be markedly prolonged, and any animal failing to negotiate the climb within 60 seconds was marked "failure." For calculating the area of the polygon, an animal failing to climb within the cut-off time was arbitrarily assigned a climbing time of 60 seconds. Although this introduced some bias into the calculation, such strong effects usually lasted only a short time and did not seriously interfere with the evaluation. The unit of area under the climbing time curve has been designated the "minute-second," and may be defined as a delay in climbing time of one second over the base line, such delay lasting for one minute. Essentially, it is the product of the intensity of the effect and its duration. This concept has been more fully explained in a previous publication(5). Simple formulae were set up for rapidly calculating the areas; the total area of polygon was designated "climbing time delay" (C.T.D.) in min-sec. One example of such a formula: when readings are taken at time intervals listed in preceding paragraph, the maximal effect occurs at 20 minutes (taking injection time as 0 time), and C. T. nearly reaches the base line at 2 hours, it can be shown that C.T.D. in min-sec $= 5(b+1.5c+2d+2.5e+3f+4.5g+6h+3i-23.5a)$, where a = C.T. before injection, and $b,c,\ldots .i$ = C.T. at 5, 10, ... 120 min. after injection. This calculation is made quickly on calculating machine, and similar simple formulae can be determined for durations of drug action other than 2 hours.

Results. Symptoms of LSD intoxication in rats. When LSD is injected intraperitoneally in fasted rats, the first sign can be observed within 1 to 2 minutes, and consists of hyperactivity. The animal explores its cage more busily than usual. After 2 or 3 minutes, this activity is interrupted momentarily while the animal violently shakes his head; this shaking may be so pronounced that it involves not only the head but the entire body. After 3 to 5 minutes, the animals undergo occasional periods when all 4 legs are flexed so that the abdomen touches the floor of cage, and the animal will crawl around on the floor. There is none of the walking backward which Woolley(1) has described in mice. After about 5 to 10 minutes, hyperactivity subsides and the animals become unusually quiet; they usually withdraw to back of cage and remain nearly motionless for prolonged period. If the cage door is opened, the difference between these animals and untreated fasted rats is very striking; the normal rats crowd to the door and eagerly sniff at the observer, while the intoxicated rats remain at furthest possible point from the door. After about 15 minutes, the animals salivate profusely.

When the LSD-treated rat is picked up at height of drug effect, he may appear to be nearly normal as long as he is being held in the hand, but if he is placed at bottom of rope, he appears to be confused. The animal may stay on electrified grid for several seconds or more, uttering a "squeak" each time the shock is applied, before he jumps onto the rope to avoid the shocks. Once on the rope, his method of climbing is different from that of a normal rat. The normal animal advances both forepaws at once, then both hindpaws, so that he appears to be literally leap-

TABLE I. Dose-Response Relationship between Lysergic Acid Diethylamide (LSD) and Climbing Time Delay (C.T.D.) in Rats Trained to Climb a Rope.

Dose LSD, mg/kg	.175	.25	.35	.5
No. of animals	5	5	5	5
C.T.D., min.-sec.	165	484	1,047	1,316
± Stand. error	35.8	85.9	222.3	297.4

Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Total	19	7,040,599		
Within groups (error)	16	2,931,284	180,205	1
Between groups	3	4,109,315	1,369,772	7.6*
Linear regression	1	3,672,129	3,672,129	20.2*
Deviations from linear regression	2	437,186	218,593	1.2†

* Highly significant $P < 0.01$. † Not significant.

Stand. dev. of single determination = 425.

Regression equation: $Y = 2032 + 2414 \log X$, in which Y = C.T.D. in minute-seconds, X dose of LSD, mg/kg.

Index of precision (Lambda) = 425/2414 = 0.15.

ing up the rope; the intoxicated animal, on the other hand, slowly advances one paw at a time. The treated animal may climb a short distance, then stop and remain motionless as though staring into space. Often, he will become so confused that he will try to circle around the rope instead of climbing, or he may climb part way up, then turn around and come down again. If he does succeed in making the climb, he hangs his head over the food cup without eating.

The extreme effects just described are seen in the majority of animals at a dose of 0.5 mg/kg, and in an occasional rat at 0.25 mg/kg; at 1 mg/kg the effects are the same and last somewhat longer.

Dose-response curve for LSD in rats. As shown in Table I, the effect of LSD in producing delay in climbing time (C.T.D.) may be measured objectively and quantitatively, and is related to log dose in the range of 0.175 to 0.5 mg/kg. The statistical analysis shows that a fairly reliable assay can be achieved with 5 animals per dose, and that within the range used, there is no significant deviation from linearity. The experiments shown in Table I were all performed on the same day. In addition to these, nine other determinations of C.T.D. have been made at the dose of 0.5 mg/kg during a period of 3 months; the mean values obtained ranged from 832 to 1861 min-sec, with an overall mean of 1230 min-sec, a value not much different from that shown in the table.

Modification of the effect of LSD by other drugs. When either 5-hydroxy tryptamine* (serotonin) or 5-hydroxy tryptophane is injected intraperitoneally 30 minutes before LSD, the effects of the latter on climbing time are strongly antagonized (Table II). Serotonin alone, produces a characteristic syndrome which differs markedly from that of LSD. The eyes become half-closed, but open wide when animals are handled. Animals withdraw to back of cage, but instead of being flat on the floor as after LSD, they tend to elevate the fore quarters and stand erect. Strong contractions of abdominal musculature may be seen after intraperitoneal injection, with extension of hind legs from time to time. Animals appear apprehensive and fearful, and react strongly to noise. The tail is occasionally whipped back and forth in a horizontal plane. When climbing, they tend to advance one paw at a time instead of leaping up the rope, but they do this fairly rapidly so that climbing time is but little prolonged. When they reach the food cup, they usually eat readily. When LSD is injected into serotonin-treated rats, the LSD-syndrome is markedly attenuated. The hyperactivity, head shaking, and crawling movements still occur but at reduced intensity; the excessive salivation is abolished. When placed on the

* Serotonin was injected as creatinine sulfate, and dose is expressed as base.

TABLE II. Antagonism of 5-Hydroxy Tryptamine and 5-Hydroxy Tryptophane to Effects of Lysergic Acid Diethylamide (LSD) in Trained Rats. Antagonist injected 30 min. before LSD. Intraperitoneal administration.

LSD			LSD + antagonist			
Dose, mg/kg	No. animals	C.T.D., min.-sec.	Antagonist	Dose, mg/kg	No. animals	C.T.D., min.-sec.	P
.5	24	1045	5-hydroxy tryptamine	.9	26	221	*
.5	5	1616	" tryptophane	5	5	446	.04
.25	5	776	" "	2	5	258	.05

* Pooled results of 5 exp.: "P" for difference between rats treated with LSD alone and LSD + antagonist varied from .05 to <.001.

rope, there is little sign of confusion, and climbing movements are well coordinated.

5-hydroxy tryptophane in the doses used did not produce any signs in rats except some fur erection and considerable scratching at dose of 5 mg/kg. There was no effect on climbing performance or C.T. When these animals were given LSD, the results were identical with those receiving both LSD and serotonin.

The antagonism of LSD with intraperitoneally administered serotonin is of especial interest, since it has been claimed that peripherally administered serotonin does not enter the brain readily, and that the effects of LSD in mice can be antagonized by serotonin only if the latter is injected intracerebrally in combination with a cholinergic drug(1). Our results are more in accord with the findings of Shore *et al.*(3) that peripherally administered serotonin produces central effects.

Neither reserpine nor α-(4-piperidyl) benzhydrol antagonized the effects of LSD (Table III). Reserpine by itself produced considerable delay in climbing, as might be expected from the depressant properties of this drug. When LSD was injected into reserpine-treated rats, the C.T.D. was much greater than with either drug alone; indeed, the figure given in the table for the higher dose of reserpine is somewhat lower than the true figure, for the experiment was terminated 3 hours after LSD when the C.T. had not yet reached the base line. The LSD-syndrome was more intense and more prolonged in these animals than in any we have seen given this dose of LSD alone. In some of the rats, the effects of LSD were still maximal 45 minutes after injection; normally, maximal LSD effects are seldom seen beyond the 20 minute reading.

The LSD-syndrome in the rats pretreated with α-(4-piperidyl) benzhydrol was identical with that seen with LSD alone. An analog of this compound, α-(2-piperidyl) benzhydrol, has been reported to block the LSD psychosis

TABLE III. Effect of Reserpine and of α-(4-Piperidyl) Benzhydrol on Action of Lysergic Acid Diethylamide (LSD) in Trained Rats.

	Treatment	No. animals	C.T.D., min.-sec.	P*
1.	LSD 0.5 mg/kg	5	1236	
2.	Reserpine 5 mg/kg p.o. × 3 da.	5	1180	
	" 2.5 " " " "	5	470	
3.	Reserpine 4 daily doses prior to LSD†			
	" 5 mg/kg, LSD .5 mg/kg	5	3348	.005
	" 2.5 " , *Idem*	5	2335	>.05
4.	α-(4-Piperidyl) benzhydrol 25 mg/kg	5	217	
5.	α-(4-Piperidyl) benzhydrol 30 min. prior to 0.5 mg/kg of LSD:			
	α-(4-Piperidyl) benzhydrol 12.5 mg/kg	5	1120	>.05
	Idem 25 "	5	1223	>.05

* P = probability of difference between drug combinations and LSD alone.
† LSD inj. 30 min. after last dose of reserpine.

in man(6). We have but a single experiment on this analog in the climbing rats; 10 mg/kg failed to block the effect of 1 mg/kg of LSD. Further experiments of the effects of this drug in LSD-treated animals are indicated.

Summary. 1) A syndrome resulting from intraperitoneal injection of LSD in rats is described. In rats trained to climb a rope, LSD produces signs of confusion and markedly prolongs climbing time. This effect on climbing time can be objectively and quantitatively measured, and is linearly related to the log dose of LSD. 2) Effects of LSD could be partially antagonized by serotonin or by 5-hydroxy tryptophane administered intraperitoneally. α-4 (piperidyl) benzhydrol did not affect the action of LSD, while reserpine intensified and prolonged the LSD-syndrome.

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Reversible Effect of Hypotonic Solutions on Growth of Influenza Virus in Tissue Cultures.* (22454)

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A previous publication from this laboratory (1) has described the inhibitory effect of potassium deficiency on growth of influenza virus in chorioallantoic (CA) membrane suspended in a medium containing the usual amounts of other inorganic salts and glucose or pyruvate. Although there was some evidence that K^+ may play a part in penetration of virus into cells, it was suggested that deficiency of this ion causes inhibition of intracellular enzyme systems concerned in virus syntheses. Previous investigations have emphasized the necessity of NaCl or other electrolytes at concentrations from 0.01 M to 0.15 M for adsorption of influenza virus to red cells(2), to mucoid hemagglutinin inhibitors, and for enzymatic action of virus on mucoid inhibitors(3). Absence or a low concentration of electrolytes resulted in diminished adsorption of influenza virus hemagglutinins to CA membrane *in vitro*(4) and similar observations had been made earlier with mouse lung and pneumonia virus of mice(5). Salt is also necessary for adsorption of phage or influenza virus to cationic ion exchange resins (6).

In most of the work just cited little effect on virus adsorption was observed unless the concentration of electrolyte in the solution was reduced to values of 0.01 M or less. In the observations to be described partial and reversible inhibition of virus growth occurred in suspensions of CA membrane with Hanks' balanced salt solution diluted to contain electrolyte between 0.04 and 0.08 M. Addition of NaCl to return the salt concentration to normal allowed growth of virus to proceed. It is believed that the inhibition of virus growth may be attributed more to reversible effects on tissue metabolism, as suggested by measurements of O_2 consumption, than to prevention of adsorption through purely electrostatic effects at the cell membrane. The relative roles of electrolyte (NaCl) and osmotic effects produced by various concentrations of glucose on growth of virus and its dissociation from tissue were investigated.

Materials and methods. Tissue suspensions of minced CA membranes from 11 or 12 day

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chick embryos were prepared as previously described(7) using shaken flasks or roller tubes. *Virus*—The PR8 strain of influenza A was added in amounts representing about 100 tissue culture infectious doses. Infectivity titrations were done in embryonated eggs, hemagglutinin titrations (HA) by the Salk method. *Balanced salt solution (BSS)*—Hanks' salt solution (without bicarbonate) was prepared at the usual concentration (100%BSS) and diluted 1:2 (50%BSS), 1:4 (25%BSS) with water. As energy sources 1 mg/ml of sodium pyruvate and 0.2 mg/ml of glucose were added to both diluted and undiluted BSS. In certain experiments with diluted BSS, NaCl was added in amounts (6 mg/ml of 25%BSS) necessary to return the electrolyte concentration to "normal." Glucose at a concentration of 25 mg/ml (0.14M) was also used for readjustment of the osmotic pressure of the 25% BSS.† Removal of excess electrolytes from the surface of the tissue was accomplished by washing the minced tissue in the concentration of BSS to be used in the particular experimental group. Tissue was suspended in about 10 volumes of solution and packed in a graduated 15 cc centrifuge tube at 1,500 rpm for 15 minutes, the supernatant removed and the processs repeated. The final volume of packed tissue‡ was used as the measure of the amount added to tissue cultures or Warburg vessels. *Viability of tissue* was evaluated by observing the extension of epithelium and fibroblasts on the glass wall of the roller tubes and by measurements of oxygen consumption in the Warburg apparatus. Unless otherwise noted the latter were done in 100%BSS with 1 mg/ml glucose for periods of 3 to 5 hours and the results expressed as μl O_2 per ml wet tissue per hour. Possible alterations of the volume of tissue by osmotic effects will be considered later.

† 25 mg/ml of glucose is taken as equivalent to 4.0 mg/ml NaCl in osmotic effect. Normal concentration of NaCl in BSS is 8 mg/ml (.138 M). KCl is 0.4 mg/ml (.018 M) and other inorganic constituents are less than 0.4 mg/ml.

‡ Measured in protein determination centrifuge tube with graduations in 0.01 ml.

Results. To confirm that no effect on adsorption occurs under the conditions of our experiments, a test similar to those previously described(4) was done with minced tissue in the presence of 25%BSS and 100%BSS both containing 0.3% serum albumin as a stabilizing agent. Tissue washed in the respective concentrations of BSS was added in amounts of 0.4 ml per 4 ml of medium and virus to make approximately 100 HA units per ml in one series and 20 per ml in another was incubated with the tissue for periods of 1, 3 and 5 hours. Virus controls were set up without tissue. After 3 hours the titers of hemagglutinins were reduced to an equal degree in 25% and 100%BSS reaching a level ½ of the controls with an inoculum of 100 HA units, while with the smaller inoculum of 20 units 90% of the hemagglutinating virus disappeared from the supernatant. At 5 hours, due to growth, hemagglutinating units in excess of those originally present appeared in the preparations with 100%BSS, while in the 25%BSS the titer was still below that at the zero hour. It appears from the results of this experiment that concentrations of electrolytes must be below those present in 25%BSS before effects on adsorption of hemagglutinins by minced tissue become measurable.

Effect of 25%BSS on early virus growth. When tissue is suspended in 25%BSS and inoculated with 100 infectious doses of virus, hemagglutinins may not become detectable before 48 or 72 hours, while they appear in the control with 100%BSS at 18 hours. Infectivity titrations on washed ground-up tissue indicate that slight multiplication of the virus has occurred by 6 hours, but the amount in 25% BSS is more than 2.5 logs below the controls. The results of infectivity titrations on tissue and supernatant fluid are shown in Fig. 1. In both 25% and 100%BSS the titer of the fluids tends to lag behind that of the tissue. Between 6 and 18 hours the tissue: fluid ratio of titers is lower in 100%BSS than in the hypotonic preparations. This may represent greater inactivation of free virus in the 25%BSS, or it may be due to delay in liberation of virus from tissue. By 24 hours the amount of virus in the tissue with 25%BSS

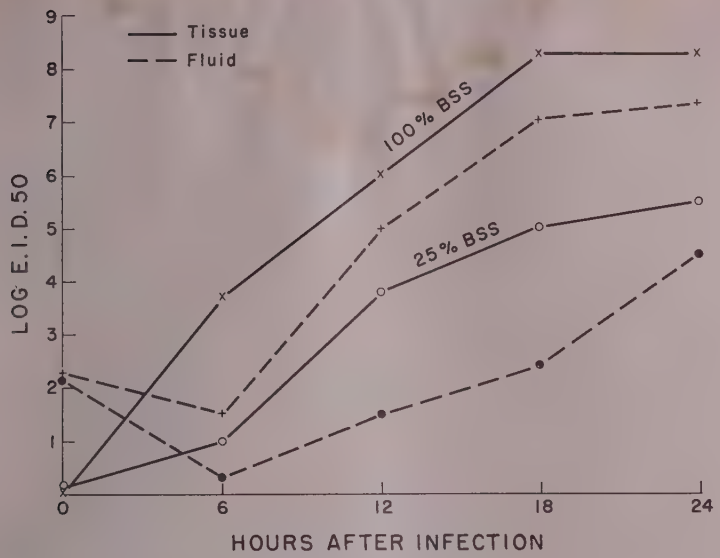

FIG. 1. Effect of 25% BSS on infectivity titers of virus in tissue and fluid. Roller tube tissue cultures.

had reached the level of the controls at 11 or 12 hours and the ratio of infectivity titers for tissue:fluid had returned to a value of approximately 10, similar to that in 100%BSS. The difference in titer of tissue between the groups with 25%BSS and the controls was maintained at about 2.5 logs.

Reversal of inhibition by late addition of NaCl. Titration of hemagglutinins at periods after 18 hours indicated the same difference in titers between 25%BSS and controls as those measured by infectivity. By 72 or 96 hours, as shown in Fig. 2, the HA titer of the controls had reached a plateau while that of

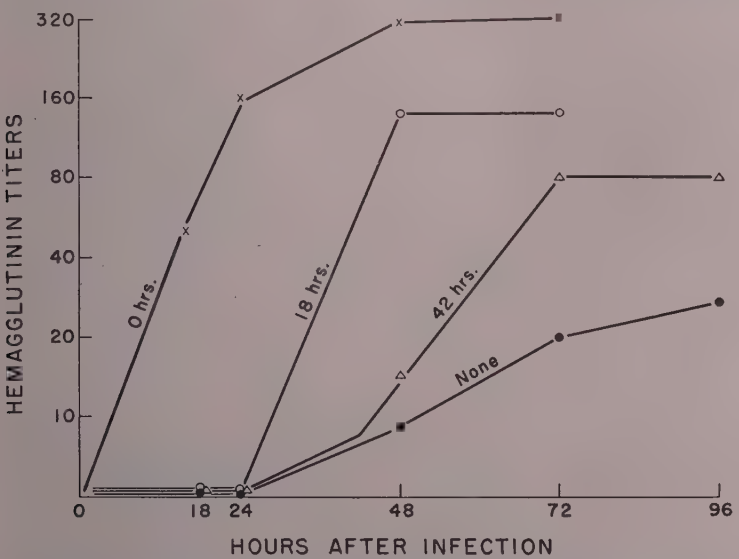

FIG. 2. Reversibility of inhibition of viral growth with NaCl. Additional amount of 6 mg/ml added to roller tube cultures in 25% BSS at times indicated on curves.

TABLE I. Effect of Medium on Growth of Virus and Tissue: Reversal by NaCl.

BSS, %	NaCl at hr	HA titer at hr			Tissue extension‡					
					Epithelium			Fibroblasts		
		24	48	72	24	48	72	24	48	72
25; 1.0 P; 0.2 G*	—	2	3	15	1+	1+	1+–±	0	0	±–0
"	18	2	90	150	±	±	1+–±	0	±	1+–±
50; 1.0 P; 0.2 G	—	12	80	150	2+	1+–2+	2+	1+–0	2+	2+–3+
"	18	38	140	280	2+	1+–2+	2+	±	2+	2+–3+
100; 1.0 P; 0.2 G	—	100	180	220	2+	2+	2+–3+	2+	2+–3+	2+–3+
25; 25 G†	—	2	32	40	2+–3+	2+	1+–2+	±	1+	1+
50; "	—	16	80	120	3+	2+	2+	1+–2+	2+	1+
100; "	—	32	80	160	2+	2+	3+	1+	2+	2+

* 1.0 mg/ml pyruvate and 0.2 glucose.

† 25 mg/ml glucose (.14M); also contains .7 mg/ml $NaHCO_3$.

‡ Relative amounts (1+, 2+, 3+) of epithelium or fibroblasts seen to migrate or grow out from the original fragments. ± indicates degeneration, round cells only.

the hypotonic preparations was still rising so that eventually a value about one-tenth of the controls was reached. With 50%BSS there was some initial delay in virus production but this was mostly overcome by 72 hours (Table I). When NaCl was added to the preparations with 25%BSS in amounts necessary to make the solution isotonic, increased titers of hemagglutinins appeared quite promptly in the supernatant fluids (Fig. 2 and Table I). Partial reversal of the effects of 25%BSS on virus growth could be obtained by adding NaCl as late as 42 hours after infection. Microscopic examination of the tissue revealed marked inhibition of the extension of epithelium and fibroblasts from the tissue fragments in the 25%BSS and growth was not appreciably stimulated by the addition of NaCl. The increased rate of virus multiplication after late addition of NaCl occurred, therefore, without observable change in the condition of the host cells. With 50%BSS the extension of epithelium and fibroblasts was somewhat delayed but not much less than in the controls and late addition of NaCl again caused little detectable change.

Partial substitution of KCl or glucose for NaCl. Use of BSS with 6 mg/ml of KCl and 2 mg/ml of NaCl resulted in growth with virus and tissue about equal to that with BSS having a normal concentration of NaCl. Higher relative concentrations of K^+ poisoned the tissue. By adding glucose to a concentration of 25 mg/ml in the 25%BSS, the osmolarity of the medium was restored to a value approximately ¾ that of normal BSS. In these experiments it was essential to add $NaHCO_3$ (0.7 mg/ml) and keep the roller tubes loosely capped in order to balance excessive acid formation. The initial pH above 8.2 gradually fell to values between 7.4 and 8.0 by the end of the experiment. The addition of glucose resulted in somewhat better virus growth as compared to that in 25%BSS alone, but inhibition was very definite at 24 hours. With high glucose, extension of epithelium was equal to that of the controls during the first 48 hours. The result suggests that osmotic effects play an important part in growth of the host cell while growth of virus is not effectively stimulated by adjustment of the osmolarity without adding more electrolyte.

Addition of calcium chloride or inorganic phosphate to 25%BSS in amounts necessary to make the concentration of these substances equal to their concentration in 100%BSS had no effect. Magnesium was present in sufficient quantity as determined by unpublished experiments of other investigators.‡

Inhibition of growth and loss of virus from tissue by changing from 100% to 25%BSS. CA membrane was infected in 100%BSS and incubated in shaker flasks until hemagglutinins became detectable in the supernatant fluid. The appearance of virus in the fluid required 18 to 24 hours. At this time the tissue

‡ R. S. Gohd personal communication.

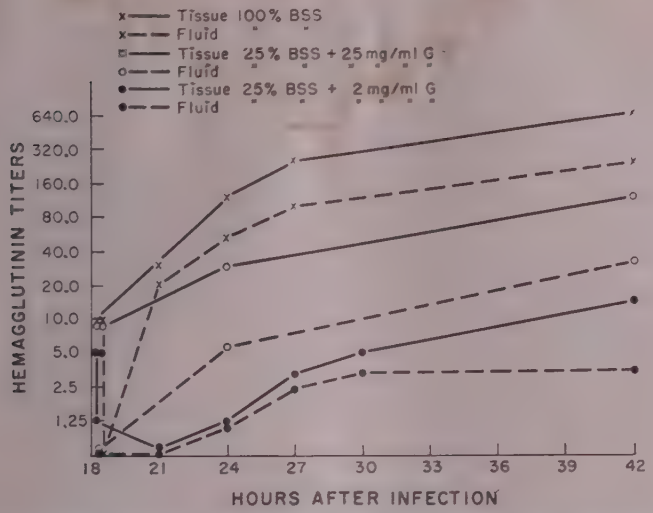

FIG. 3. Infected tissue incubated for 18 hr in 100% BSS, washed and changed to fresh solutions as indicated. In this experiment 100% BSS contained 1 mg/ml pyruvate and 0.2 mg/ml glucose.

washed with 100%BSS and ground in buffered normal saline to make a 10% suspension gave an HA titer approximately equal to that in the fluid. When infected tissue was washed twice with 25%BSS and then ground in buffered normal saline the HA titer was found to have dropped to 1/2 to 1/16 that in tissue washed with normal BSS. Measurements of the volume of tissue revealed no significant change such as might be expected from possible cytolysis or osmotic swelling. Further incubation of the samples of washed infected tissue with 100% and 25%BSS was then done in flasks or roller tubes. The resulting effects on HA titers are depicted in Fig. 3. Tissue washed with 100%BSS and returned to this medium showed a continued increase in HA titer and there was also a prompt recovery of HA in the supernatant fluid. When the tissue was placed in 25%BSS there was a loss of virus from the tissue followed by a little increase, and accompanied by a partial recovery of hemagglutinins in the fluid. When the washed tissue was returned to 25%BSS containing 25 mg/ml of glucose the virus continued to increase, but at a rate intermediate between that of plain 25%BSS and 100%BSS. Correction of the osmotic effects by means of glucose instead of NaCl did therefore favor the continuous growth of virus and prevented its initial loss from tissue. In another experiment all infected tissue was first washed in 100%BSS and then samples were placed in 100, 50, 35 and 25%BSS. The results of HA titrations on the tissue are shown in Fig. 4. It may be seen that the loss of hemagglutinating virus from the tissue was a progressive process which was related to the concentration of salt. In tissue pre-incubated for 18 hours and then placed in 25%BSS the HA titer drops promptly to less than 2. With 35% and 50%BSS the titers remained below the controls for 3 hours and then showed a drop. The remaining lines in Fig. 4 illustrate a similar course of events in tissue pre-incubated for 24 hours in 100%BSS so that the virus titer was approaching a maximum at the time of change to lower concentrations of BSS. In all instances a loss of virus was evident in tissue incubated with the hypotonic BSS.

Effect of hypotonic BSS on tissue respiration. Measurements of O_2 uptake were done in the Warburg apparatus with fresh tissue in 25% and 50%BSS, and also on tissue which had been pre-incubated for various periods of time in hypotonic BSS and then placed in 100%BSS for the O_2 measurement.

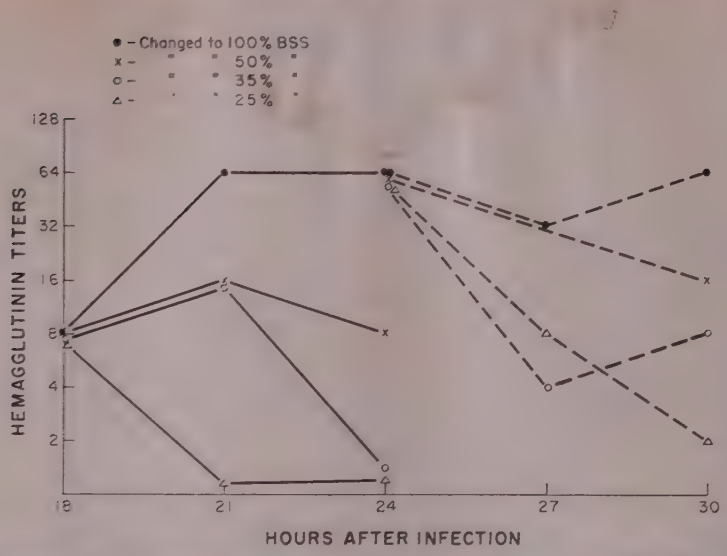

FIG. 4. Hemagglutinin titers of virus in tissue after pre-incubation in 100% BSS and change to diluted BSS as indicated at 18 hr (solid lines) or 24 hr (broken lines).

In general 50% BSS produced little change in the rate of O_2 consumption either with fresh tissue or with tissue incubated in this solution for 48 to 72 hours. On the other hand, the respiration of fresh tissue placed in 25% BSS was reduced almost immediately to about one-third of the rate with control tissue in 100% BSS. Since the volume of tissue was measured before placing it in the Warburg vessels these differences cannot be attributed to errors introduced by osmotic swelling during the course of the measurement of O_2 consumption. The reversibility of this inhibition of respiration is indicated by several lines of evidence based on the data in Table II. Addition of NaCl from the side arm of the Warburg vessel in amounts necessary to reestablish isotonicity at 3 hours after the start of the experiment caused a partial reversal within a short period of time. Although in a 24 hour period the controls showed a descending rate of respiration (8), the group with added NaCl reached 65% of the controls at this time. Warburg measurements on tissue

TABLE II. Oxygen Consumption after Treatment of Tissue with Hypotonic Solutions.

Pre-incubation	Medium in Warburg vessels	Rate O_2 uptake as % of controls at age of tissue in hr: 3	6	24	48
None; fresh tissue	25% BSS; 1.0P; .2G*	30	30	33	—
Idem	25% BSS; " " + NaCl at 3 hr	30	50	65	—
"	25% BSS; 1.0P; 25G*	52	49	—	—
"	50% BSS; 1.0P; .2G*	80–95†	—	—	—
25% BSS, 24-48 hr‡	100% BSS; 1.0G	—	—	70–100	60–90
25% BSS + 25G, 24-48 hr	*Idem*	—	—	90	70
50% BSS, 24-48 hr‡	"	—	—	70–100	60–90
100% BSS, 24 hr; then 25% BSS§	"	—	—	50–75	10–20
100% BSS, 18 hr; then 25% BSS + 25G	"	—	—	75	60
100% BSS, 24 hr; then 50% BSS	"	—	—	100	90

* P = pyruvate as mg/ml, G = glucose as mg/ml, corresponding controls with 100% BSS and equivalent glucose concentration.

† Double figures indicate low and high of range in several experiments.

‡ Controls pre-incubated with 100% BSS; 1.0 P + 0.2 G in all series.

§ Tissue washed with 25% BSS, and further incubated in 25% BSS. Corresponding controls washed and reincubated in 100% BSS.

in 25% BSS containing 25 mg/ml glucose revealed a marked depression of respiration. In this medium the osmotic pressure was intermediate between that of 50% BSS and normal but as shown in Table II the depression of O_2 uptake with 50% BSS was much less. In the controls with 100% BSS and 25 mg/ml glucose some substrate stimulation of respiration, as compared with the O_2 uptake of tissue in BSS containing only pyruvate, was observed. When fresh tissue was put in 25% BSS in roller tubes or shaken flasks and incubated for 24 to 48 hours, Warburg measurements done with this tissue in 100% BSS with 1 mg/ml glucose showed rates in some instances were equal to but usually slightly less than those for control tissue pre-incubated in 100% BSS. Since tissue kept in 25% BSS in the Warburg vessels for 24 hours showed a markedly depressed respiration these results suggest that return of the tissue to normal conditions at 24 to 48 hours results in reversal of inhibition of respiration. Another possible interpretation would be that the fresh tissue is able to adapt itself to the hypotonic solution after a period of incubation. Indeed some evidence for this is found in the observation that small spherules of epithelium (9) often were formed in the hypotonic BSS and this might allow some concentration of electrolytes in the interior medium of these structures. Measurements of the volume of tissue recoverable by packing in graduated centrifuge tubes revealed no difference between the effects of incubation with 25% BSS and those with 100% BSS until 3 days when the tissue volume in 100% BSS had decreased by autolysis to about half of that originally present, while the volume of tissue in the 25% BSS was about ¾ of the original. Combined autolysis and swelling might account for this result.

Tissue pre-incubated for 24 hours in 100% BSS and then washed with 25% BSS and reincubated in the hypotonic solution showed definite signs of irreversible damage. The washing with 25% BSS in itself resulted in a decrease in rate of respiration as measured in 100% BSS although no swelling of the tissue was observed during this treatment. Further incubation resulted in a very marked depression of respiration as contrasted with the oxygen consumption of tissue which was placed in 25% BSS when fresh. Tissue incubated with 25% BSS from the beginning then washed at 24 hours and reincubated in this same medium or in 100% BSS did not show the depression of respiration; another observation which indicates that fresh tissue can show a certain degree of adaptation to the hypotonic medium.

Infection with virus seemed to have no relation to the observations on tissue respiration just described. The depression of respiration by 25% BSS was about the same in degree in normal and infected tissue.

Irreversible damage was less marked when tissue pre-incubated in 100% BSS was placed in 25% BSS containing 25 mg/ml glucose (next to last line of Table II). This suggests that osmotic effects rather than lack of NaCl *per se* play an important part in the tissue deterioration under these conditions. However, pre-incubated tissue placed in 50% BSS showed less depression of respiration than that put in 25% BSS with 25 mg/ml glucose.

Discussion. At the concentrations of electrolyte in the range 0.04 M to 0.08 M used in these experiments there was no directly measurable inhibition of the adsorption of virus to tissue and this factor was probably negligible in the observed retardation of growth. An indirect role of NaCl or other electrolytes in aiding irreversible attachment or penetration of the cell membrane by virus seems possible. For example, Garen and Puck (10) found that when T_2 bacteriophage was attached to *E. coli* B in the presence of 0.04 or 0.02 M NaCl the virus could be eluted with distilled water and the host cells survived. Attachment in the presence of 0.1 M NaCl, on the other hand, was not reversible and the host cells were killed. Furthermore, absence of electrolyte apparently prevented multiplication of virus because nutrient agar without NaCl permitted growth of *E. coli* B, but not of the associated phage. The authors expressed the opinion that the irreversible attachment of T_2, as a step in infection, repre-

sented an enzymatic establishment of covalent linkages, while the reversible attachment was probably electrostatic in nature. The curves in Fig. 1 indicate that multiplication of influenza virus in the tissue is retarded from the start of the experiment. This could be attributed either to unsuccessful penetration of the tissue by a large proportion of the adsorbed virus particles or to delayed reproduction of the intracellular virus.

Dissociation of virus from heavily infected tissue as the result of exposure to hypotonic medium is clearly indicated by the data in Fig. 3 and 4, but it is not known that a similar effect occurs during the initial stages of infection. The results are reminiscent of the dissociation of pneumonia virus of mice from lung tissue or red blood cells in the presence of distilled water or 0.25 M dextrose respectively(5). Davenport and Horsfall found that the pneumonia virus failed to dissociate from red blood cells in 0.25 M glucose, only when the pH was below 7.0. In our own experiments loss of influenza virus from tissue occurred in diluted BSS with electrolyte concentrations ranging from 0.04 to 0.08 M and this dissociation (Fig. 3) was apparently prevented by adding 0.14 M glucose to 25%BSS buffered at a pH near 8.0. Since the osmolarity of the solution was increased by this addition but not the electrolyte content, it seems evident that the observed dissociation of virus from tissue in this case is at least in part due to osmotic effects. Gross plasmolysis was not observable in 25%BSS.

Although reduction of the salt concentration to half of the normal level produced little significant change in the respiratory metabolism of chorioallantoic tissue, solutions more hypotonic than this caused definite inhibition of O_2 uptake. This effect with fresh tissue was for the most part reversible, but with tissue pre-incubated in 100%BSS for 24 hours, irreversible changes occurred on exposure to 25%BSS. Neither of these effects was completely overcome by the addition of 0.14 M glucose (Table II), and although the resulting osmolarity was greater than that of 50% BSS, the tissue respiration was not preserved as effectively. It will also be noted (Table I) that virus growth in 0.04 M electrolyte (25%BSS) plus 0.14 M glucose was slower than in 0.08 M electrolyte. These observations suggest that lack of electrolytes (as NaCl or KCl) by itself and apart from the osmolarity of the solution has a depressive effect on tissue metabolism and probably virus synthesis. There was no clear-cut relation between metabolism, proliferation of tissue, and growth of virus.

The relation of these observations to the effect of electrolytes and the osmotic pressure of solutions on mitochondria should be considered. Isolated mitochondria maintain their normal morphology only when the solution is hypertonic(11,12) with 0.88 M sucrose, while physiological NaCl results in rounded swollen forms not only with free mitochondria, but also with mitochondria in suspended intact liver cells(11). Respiration of free mitochondria is greatly depressed in 0.15 M NaCl and slightly in 0.15 M KCl. It may be, therefore, that in simple salt solutions such as those used in our tissue cultures the intracellular mitochondria are already abnormal and may undergo additional changes in hypotonic solutions. This might result in depressed respiration or oxidative phosphorylation, such as accompanies morphological changes in isolated mitochondria exposed to hypotonic solutions or distilled water(12). More detailed cytochemical studies would be required to establish this, but in addition there is the possibility that other unknown changes may be occurring in the cell membrane or in other components of the cytoplasm.

It is evident that effects on virus growth such as those revealed in the present study cannot be attributed simply to factors of adsorption or elution, but that reversible effects of electrolytes or osmotic pressure on tissue metabolism must also be considered.

Summary. 1. Growth of virus is inhibited by exposure of infected chorioallantoic membrane to Hanks' balanced salt solution diluted 1:2 or 1:4. This occurs either at the beginning of the experiment or after a 24 hour period of virus multiplication. Restoration of isotonicity with NaCl up to 42 hours after in-

fection results in reversal. 2. NaCl when added late, although it stimulates virus growth, does not increase tissue proliferation. Glucose 0.14 M in 25%BSS permits almost normal tissue proliferation, but does not significantly stimulate virus synthesis during the first 24 hours. 3. Loss of virus from heavily infected tissue occurs on exposure to 50, 35, or 25%BSS. This is prevented by adjusting the solution to near normal osmolarity with glucose. Tissue respiration is markedly inhibited by 25%BSS but not by 50%BSS. 4. The relative roles of electrolytes and osmotic properties of the solution in virus attachment, penetration, and synthesis and on tissue respiration are considered.

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Anesthesia: LIV. Effects of Viadril and Some Water-Soluble Steroids on Brain Oxidative Phosphorylation.* (22455)

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Although claims of "anesthesia" produced by steroids have been made previously(1,2-5), Viadril, a new water-soluble steroid, represents the first compound of this type to evoke anesthesia with a safety margin adequate for human use(6). A survey of the effects of various hypnotic and anesthetic drugs on the coupling between oxidative phosphorylation and respiration in rat brain mitochondria(7) prompted the extension of this study to Viadril. Other water-soluble steroids lacking anesthetic action were tested for comparison.

Methods. The methods used for measurement of the ratio of oxidative phosphorylation to respiration have been described more fully elsewhere(7,8). Briefly, the ratio is measured as the inorganic phosphorus uptake in micromoles of phosphorus divided by the microatoms of oxygen consumed by rat brain mitochondria which were isolated in 0.25 M sucrose containing 0.001 M disodium Versenate. The final concentrations in the medium used were: pH 7.4 phosphate buffer 0.020 M; potassium chloride 0.050 M; potassium fumarate 0.002 M; sodium pyruvate 0.013 M; ATP 0.0025 M; glucose 0.028 M; hexokinase 6.25 mg/cc; disodium Versenate 0.001 M; sodium fluoride 0.012 M; magnesium chloride 0.008 M; and was incubated at 20° for 30 minutes following a 10 minute equilibration. Viadril (21-hydroxy-pregnanedione), hydrocortisone, and pregnenolone were all employed in the form of their sodium succinate salts. The sodium salt of dehydrocholic acid was used.†

* Supported by grant from Ohio Chemical & Surgical Equipment Co., (Division of Air Reduction Co., Inc.), New York City.

† These compounds were kindly supplied by Dr. Michael Carlozzi of Pfizer Laboratories.

TABLE I. Effect of Steroid Derivatives on Oxidative Phosphorylation of Rat Brain Mitochondria.

Drug	Conc., mM	Avg control P	O	P/O	Avg experimental P	O	P/O	P/O % change	No. of flasks
Viadril	.05	15.4	4.99	3.09	13.7	5.41	2.54	−17.8	3
	.10	14.4	4.59	3.12	11.5	4.59	2.51	−19.6	5
	.25	14.9	4.48	3.33	10.0	4.04	2.48	−25.6	2
	.50	15.4	4.85	3.18	1.0	2.50	.40	−87.5	3
Hydrocortisone sodium succinate	.104	11.5	3.99	2.89	13.7	4.78	2.87	− .07	5
	.208	11.5	3.99	2.89	14.2	5.36	2.65	− 8.3	6
	.416	11.5	3.99	2.89	14.1	4.77	2.96	+ .24	5
Pregnenolone sodium succinate	.114	14.4	4.81	3.0	10.6	5.20	2.04	−32.0	3
	.228	14.4	4.81	3.0	3.9	3.51	1.11	−63.0	3
	.455	14.4	4.81	3.0	2.8	4.49	.62	−79.5	3
Dehydrocholic acid	.125	11.3	3.65	3.10	9.75	3.29	2.96	− 4.5	2
	.25	11.3	3.65	3.10	10.15	3.18	3.20	+ 3.2	2
	.50	11.3	3.65	3.10	5.5	2.55	2.16	−30.4	2

Results. Table I sets forth the data obtained for several concentrations of each agent. Both Viadril and a closely-related pregnenolone derivative devoid of anesthetic activity produced a substantial uncoupling of oxidative phosphorylation from respiration. High concentrations of dehydrocholic acid exhibited a slight effect and hydrocortisone no action.

Discussion. Pregnenolone produced uncoupling of oxidative phosphorylation and is devoid of anesthetic activity. This lack of correlation occurs in the relationship of uncoupling to anesthetic or hypnotic activity for other narcotic drugs(7,8). In this group of drugs the most potent uncoupling agents were Viadril and pregnenolone, of which only Viadril exerts anesthetic action. Murphy *et al.* (9) reported the range of anesthetic doses in the majority of patients to be 1.0-1.5 g. Using the upper figure, an estimation of the expected concentration in extracellular body water would be 10 mg % (0.22 mM) for a 75 kg person. This plasma level should produce significant depression of P/O ratio *in vitro* as indicated by the results in Table I.

Difficulties of solubility discourage the extension of these studies to other steroids claimed by Selye to have central depressant action. A previous paper from this laboratory questions the suitability of the word "anesthesia" for the effects of the steroids used by Selye(10).

Conclusion. 21-Hydroxypregnanedione sodium succinate and pregnenolone sodium succinate produce significant uncoupling of oxidative phosphorylation from oxygen consumption. Dehydrocholic acid was less effective. Hydrocortisone sodium succinate was ineffective.

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Plasma Calcium, Magnesium and Protein of Viviparous Colubrid Snakes During Estrous Cycle. (22456)

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During the season of rapid follicular growth in cod and puffer fish(1), clawed toad(2), and in many species of birds(3), increases in concentration of plasma calcium have been observed. At this same stage of the estrous cycle, a phosphoprotein has been detected in the plasmas of a trout, a turtle, and a number of species of birds(4). This paper presents evidence concerning plasma changes which occur in viviparous colubrid snakes during estrus.

Materials and methods. Specimens of 10 kinds of water snakes, *Natrix,* and 14 kinds of garter snakes, *Thamnophis,* were collected locally or were obtained from collectors in other areas of the United States. Animals were fasted for one week and then anesthetized with ether and bled from a cardiac puncture. Blood was collected in heparinized tubes. Calcium plus magnesium was determined directly upon a plasma sample; calcium was measured upon an oxalate precipitate of plasma; magnesium was calculated by taking the difference between the two values. A modification of Schwarzenback's ethylenediamine-tetraacetic acid method was used in these analyses(5). Plasma protein was determined with a biuret method(6). Reproductive stages of animals were determined by direct observation of reproductive organs and embryos.

Results. Average plasma levels of calcium, magnesium, and total protein are given in Table I. The concentration of calcium and magnesium of nonestrous females, males and immature specimens differs very little. The plasmas of females of both genera studied have higher protein concentrations than those of males during all seasons. Average calcium and protein levels are higher in *Natrix* than in *Thamnophis.* No distinct seasonal differences occur in calcium, magnesium, or total protein concentrations of male specimens. In contrast to males, marked increases in all three substances accompany estrus in females of both genera. The increase in calcium may be as much as 10 times or as little as twice as great as the increase in magnesium. The rise in total plasma protein of estrous females averages about 25% in *Thamnophis* and about 15% in *Natrix.* The absolute, average increase is about 1 g/100 ml of plasma in both genera. Preliminary observations of paper electrophoretic patterns of plasma proteins indicate that in some of the species studied a specific fraction is associated with this increase in total protein. Plasma protein concentration of estrous females is less variable than plasma calcium or magnesium levels.

To show the time relationships of these plasma changes, the sum of the concentrations of calcium plus magnesium in plasma of individual specimens of female garter snakes relative to their stage of the estrous cycle is shown in Fig. 1. Calcium plus magnesium is of low concentration until the period of rapid yolk deposition in spring. The initiation of this process is noted when the translucent follicles characteristic of anestrous snakes become opaque and begin to enlarge. When these follicles have about doubled their anestrous size, the plasma of the snake becomes slightly opalescent and contains an increased concentration of alkaline earth elements in comparison to values characteristic of other stages of the estrous cycle. Plasma level of calcium plus magnesium increases steadily as the follicles grow and attains a concentration of between 10 and 15 mM/l during the late stages of yolk deposition. Most extreme values are found in animals bled about the time

TABLE I. Plasma Composition Viviparous Snakes.

	Calcium, millimoles/l			Magnesium, millimoles/l			Protein, g/100 ml		
	No. of animals	Mean	Range	No. of animals	Mean	Range	No. of animals	Mean	Range
Thamnophis									
Adult ♀ (nonestrous)	33	3.16 ± .52*	1.7- 4.4	33	1.64 ± .58*	.8- 3.6	38	3.95 ± .72*	2.50-5.40
Adult ♀ (estrous)	19	12.29 ± 9.16	4.0-40.1	19	4.43 ± 3.40	1.0-15.9	19	5.02 ± 1.01	3.25-7.30
Adult ♂	16	3.11 ± .53	1.7- 4.0	15	1.41 ± .37	.8- 2.5	21	3.60 ± .68	2.33-5.45
Immature ♂ & ♀	3	3.17 ± .28	2.8- 3.5	3	2.03 ± .57	1.3- 2.7	9	3.18 ± .42	2.33-3.62
Natrix									
Adult ♀ (nonestrous)	21	3.44 ± .40	2.7- 4.2	19	1.49 ± .57	.8- 3.1	17	4.88 ± 1.13	2.85-6.90
Adult ♀ (estrous)	13	8.35 ± 5.50	4.0-26.0	13	2.20 ± 1.17	.8- 4.4	14	5.87 ± .76	5.03-7.50
Adult ♂	33	3.48 ± .45	2.6- 4.5	32	1.64 ± .56	.9- 3.2	34	4.65 ± .95	2.20-6.60
Immature ♂ & ♀	13	3.35 ± .47	2.6- 4.0	14	1.64 ± .44	1.1- 2.5	12	4.64 ± .73	3.20-5.70

* Stand. dev. from mean.

FIG. 1. Changes in calcium plus magnesium in plasma of female garter snakes during various stages of their estrous cycle. Each dot represents data from a freshly collected specimen. Duration of estrous stages is estimated from observations on *T. sauritus* and *T. elegans*. Data upon *T. sirtalis* have been adjusted to correlate with the timing of these species.

of ovulation. Within 2 to 3 days following ovulation, however, the opalescence of the plasma disappears and the protein and calcium plus magnesium concentrations fall to nonestrous levels.

Increased calcium and magnesium were found in the plasma of all snakes sampled during estrus. Such high values were observed in 5 kinds of garter snakes and in 4 kinds of water snakes (*T. sauritus proximus, T. sirtalis tetrataenia, T. s. sirtalis, T. elegans terrestris, T. e. gigas, N. sipedon confluens, N. cyclopion floridana, N. c. cyclopion, N. r. rhombifera*). The most extreme value of calcium plus magnesium in plasma, 46.8 mM/l, occurred in a specimen of *T. s. sirtalis* which had just ovulated. Adult females of these genera occasionally skip a reproductive season. Such adult females have low plasma calcium and magnesium during the spring period when elevated values are characteristic of females with active ovaries.

Discussion. To our knowledge the magnitude of the estrous increase in calcium, magnesium and in total plasma protein in these snakes exceeds by far any such change reported in other vertebrates. An elevation in total plasma protein of chickens during the

egg laying season has been reported by some investigators, but this reported rise is questioned by others(7). Estrogens will induce a rise in plasma calcium in male birds and in females during anestrus(3). The rise in plasma calcium of birds(3) during estrus is greater than increases found in vertebrates previously studied(1,2) but is still less than half the average concentration characteristic of these snakes.

As the eggs of viviparous snakes are not covered by a calcareous shell, it remains to be determined whether the estrous rise in calcium and magnesium represents a transport of these minerals to the developing follicles or whether the phenomenon is merely a physiologic response carried over from forms which used such inorganic material in egg shell formation. Follicles of birds develop in sequence and ovulation is at the rate of one egg/day or less. Viviparous snakes, unlike birds, develop large numbers of follicles simultaneously, reaching a maximum of 85(8). These follicles progress at approximately the same rate and are ovulated within a few minutes or at most a few hours of each other. The great metabolic demands of such large numbers of simultaneously developing follicles may be related to the greatly elevated protein, calcium and magnesium levels in the plasma of these snakes during estrus. The sharp decrease in these substances following ovulation probably is correlated with the uniform time of ovulation.

Summary. A marked increase in concentrations of calcium, magnesium and total protein occurs in plasma of female specimens of viviparous snakes during estrus. The magnitude of these estrous changes greatly exceeds any such data reported for other vertebrates. Following ovulation these plasma values rapidly fall to concentrations characteristic of nonestrous animals.

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Relationship of Blood Pressure to Mortality in Chickens.* (22457)

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Mortality losses in poultry flocks during the first laying year range from 20 to 40%. Autopsy reports indicate that 50% or more of these deaths result from non-specific disorders or undetermined causes(1-4). Whether or not any portion of the mortality was attributable to circulatory disturbances has not been ascertained. Sturkie, Weiss, and Ringer (5) previously determined systolic blood pressures on a large population of chickens, ranging in age from 10 to 54 months. At any age the pressures were widely distributed, but no attempt was made to relate the level of pressure to mortality, which is the object of this study.

Procedure. Arterial blood pressures were determined on hens hatched in 1950, 1952, and 1954 and on males in 1954 by an indirect method(6). Three consecutive determinations per bird were made at 7 to 10 months of

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TABLE I. Range of Systolic Blood Pressure for High, Median and Low Pressure Birds.

	♀			♂
	1950	1952	1954	1954
Pressure	(Range in mm Hg)			
High	>141	>151	>176	>191
Median	121–140	130–150	146–175	161–190
Low	90–120	90–129	90–145	120–160
Mean	128.0	141.0	160	180

age and the values averaged. Records of mortality and egg production were kept until the birds were approximately 19 months of age. Thus, the records were based on a period of 9 to 12 months. The birds hatched in 1950 were of one strain of White Leghorns, but those hatched in 1952 comprised 3 strains (2 unrelated strains of White Leghorns, and one crossbred). The mean pressures and distribution of pressures for the 3 strains were not significantly different. The 1954 birds represented the first generation progeny of strains selected for high and low blood pressure. When birds of the 2 strains were pooled, the frequency distribution of pressures was similar to that for the 1950 and 1952 birds. Birds which died during the test period were autopsied by C. B. Hudson and D. C. Tudor of the Animal Pathology Department.

Results. Frequency distribution of blood pressure. The birds were divided into 3 blood pressure groups: high, median and low as shown in Table I. The increase in mean systolic pressure over the years reflects the effects of environment and aging. The 1954 birds were 3 months older than those of previous years when determinations were made, and we have shown(5) that blood pressure may increase significantly in this period of time. The data confirm our previous findings (5) that blood pressure is significantly higher in the male.

Table II shows the numbers and percentages of birds falling into each of the pressure groups. Pooled figures reveal that approximately 30% of the birds have high pressures, 30% low pressures and 40% median pressures.

Relationship of mortality to blood pressure. It is apparent that mortality (Fig. 1) has been consistently higher over the years in the hypotensive birds. Since in most groups the number of birds which died was less than 100, it was considered desirable to convert the percentage figures into angles in accordance with the formula: angle = arc sin $\sqrt{\text{percentage}}$ (Snedecor, 7). An analysis of variance revealed a statistically significant difference between the pressure groups ($P = .04$). Based upon standard errors, the differences between the hypotensive and other groups were significant, but not between the median and hypertensive birds.

Age at death. The mean ages at death were as follows: high pressure birds, 397 days; median pressure, 437 days; and low pressure, 426 days. An analysis of variance revealed the differences to be not statistically significant.

Cause of deaths. The cause of the higher mortality in the hypotensive birds could not be discerned from autopsies. The distribution of diseases and disorders was similar in the 3 groups.

Blood pressure and egg production. It appeared possible that because the hypotensive birds experienced a higher mortality, their reproductive performance (number of eggs laid) might be impaired. Thus, egg production of the birds dying and of those surviving the test period was compared with blood pressure. The mean numbers of eggs and standard errors for the high, median and low pressure birds were 189.6 ± 4.02, 197.6 ± 3.40, and 188.8 ± 5.04 respectively. The percentages of egg production (from first egg laid to death) were 45.8, 52.7, and 45.0 for the high, median and low pressure birds respectively. None of the differences was statistically significant.

TABLE II. Percentages of Birds Comprising High, Median and Low Pressure Groups.

Year	Total birds	High No.	High %	Median No.	Median %	Low No.	Low %
♀							
1950	50	13	26.0	17	34.0	20	40.0
1952	112	35	31.2	43	38.4	34	30.3
1954	155	43	27.7	68	43.9	44	28.4
Totals	317	91	28.7	128	40.4	98	30.9
♂							
1954	158	37	23.4	74	46.8	47	29.7

FIG. 1. Mortality to 19 mo of age of high, median and low blood pressure chickens. For numbers of birds involved, see Table II.

Discussion. The data reveal a significantly higher mortality in the hypotensive birds, but the hypertensive birds experienced no higher mortality than those with average or median pressures. Body weights and reproductive performance were not different among the pressure groups. That the cause of the higher mortality of the hypotensive birds could not be discerned from gross autopsies, is not surprising, since routine autopsies by poultry pathologists fail to account for an appreciable number of chicken deaths. More detailed studies involving the hemodynamical sequelae of hypotension and its effects, are required.

Summary. Systolic blood pressures were determined on adult chickens at 7 to 10 months of age in 1950, 1952 and 1954. Records of mortality and egg production were kept until the birds were approximately 19 months of age. The birds were divided into three pressure groups: high, median and low. Pooled figures for the three years show that approximately 30% of the birds had high pressure, 30% low pressure and 40% median pressure. The mortality in the hypotensive birds was nearly twice that of the other pressure groups. There were no significant differences in the body weights or egg production between birds of the groups. Gross autopsies did not reveal the cause of the higher mortality in the hypotensive birds.

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Augmentation and Depression of Estriol-Induced Growth of the Uterus By Progesterone.* (22458)

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Progesterone is an important regulator of proliferation of cells whose growth is stimulated by phenolic estrogens; it augments the growth of certain tissues and depresses growth of others. Injected alone, progesterone has no effect on growth of the genital tract(1). When estrone is administered to the ovariectomized rat together with progesterone, the growth of the mammary gland is enhanced while that of the uterus is inhibited. It was found in the present experiments that the action of progesterone on growth of the uterus is different when it is administered with estriol instead of estrone. The effects of progesterone on estriol-induced uterine growth are biphasic, consisting of enhancement of growth followed by depression, depending on the dosage of estriol which is employed.

Methods. The experiments were conducted on 560 albino rats which were maintained under standardized climatic and dietary conditions(2). There were 5 rats in each group at every dose level and the experiments were repeated 5 separate times. Varying quantities of estrone or estriol were administered alone or combined with 1 mg of progesterone. The dosage of steroids refers to the amount injected daily. It had been found earlier(3) that 0.1 mg of progesterone is the smallest quantity to cause maximal depression of estrone-induced growth of the uterus of hypophysectomized rats. The steroids were dissolved in ethyl alcohol which was diluted with sesame oil to make the final alcoholic concentration 10%. Hypophysectomy was performed at age 24 days and the steroids were injected (0.2 ml) subcutaneously daily from age 38 to 44 days. Necropsy was carried out at age 45 days when the uterus was excised, blotted and weighed on a torsion balance. The nitrogen content of the uterus was determined by a micro-Kjeldahl technic. Growth is defined as an increase both of weight and of nitrogen content. It was found in each case that the nitrogen concentration of the uterus was 2.52 ± 0.08 mg per 100 mg moist weight so that the simple determination of the weight of the uterus had significance.

Results. The results of the 5 experiments were essentially similar. With a dose level of estrone (0.05 μg), just sufficient to initiate growth of the uterus, progesterone exerted no detectable effect on such growth. With increased dosage of estrone (0.1 μg) progesterone exerted an inhibitory effect (Fig. 1) which reached its maximum (32%) when the amount of estrone was 0.15 μg. No augmentation of estrone-induced uterine growth by progesterone was observed.

With reference to the growth of the uterus induced by estriol alone (Fig. 2) progesterone augmented the growth induced by small amounts (1-5 μg) of estriol and inhibited the growth caused by larger doses (25-50 μg). At the 10 μg dosage level of estriol, progesterone did not modify the growth of the uterus. The maximum augmentation of estriol-induced uterine growth by progesterone was 18%; the maximum inhibition was 24%.

Discussion. Estriol is an impeded estrogen (2) belonging to a small class of 1, 3, 5(10)-estratrien-3-ol derivatives with oxygenated functions at specific molecular sites. The impeded estrogens have common physiologic properties that differ from those induced by the majority of estrogenic compounds. 1. Estriol is more potent than estrone(4) in causing opening of the vaginal plate of infantile rats but is less active in evoking vaginal cornification. 2. Whereas estriol in small

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quantity initiates uterine growth, a considerable increase of dosage augments this growth only slightly(5). 3. Hisaw *et al.*(6) discovered that estriol can reduce the effectiveness of estrone in promoting uterine growth when these steroids are injected together.

Evidence has been presented that these physiologic properties of estriol are due to the interaction of its 16α-hydroxyl group with that specific protein surface of the uterine cell which is of critical importance in promoting growth. It would appear from the present experiments that progesterone can block the inhibitory action of this hydroxyl group when small amounts of estriol are present and so enhance the growth promoting activity of the phenolic group in the molecule.

Conclusions. 1. Whereas progesterone inhibits the growth promoting action of estrone

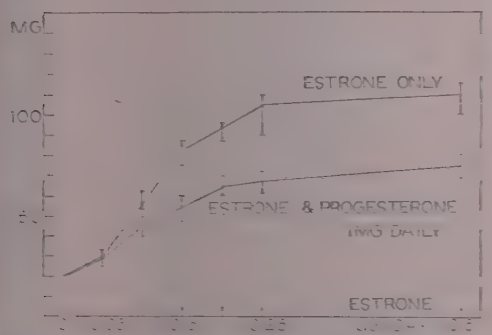

FIG. 1. Effect of progesterone, 1 mg daily, on growth of uterus induced by estrone. Ordinates: wt of uterus. Abscissae: daily dosage of estrone.

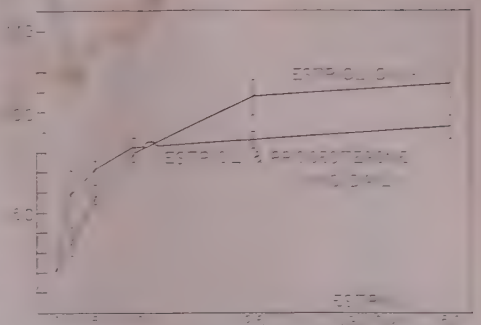

FIG. 2. Effects of progesterone, 1 mg daily, on growth of uterus induced by estriol. With smaller quantities of estriol, growth is enhanced but with larger quantities it is inhibited. Ordinates: wt of uterus. Abscissae: daily dosage of estriol.

on the uterus of hypophysectomized rats, its effects on estriol-induced growth are biphasic. 2. Progesterone depresses uterine growth evoked by large quantities of estriol but *enhances* growth promoting action of small quantities of this impeded estrogen.

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Anticonvulsant Effect of Morpholinethylmorphine. 22459

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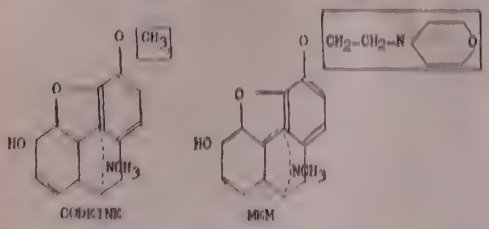
Morpholinethylmorphine (MEM)* is an amino alkyl ether of morphine resulting from substitution of morphine's phenolic hydrogen by the morpholinethyl radical. It was prepared by Chabrier, *et al.*(1) and described as an anti-tussive agent which possessed neither the convulsant effect nor the smooth muscle stimulating action of codeine. The close structural relationship of MEM to codeine

* Obtained from Dr. R. Giudicelli, Dausse Laboratories, Paris, France.

(methylmorphine) is shown below.



The present investigation is concerned with a comparison of the central depressant effect of MEM with that of codeine, some synthetic morphine analogues (methadone isomers), Miltown, and chlorpromazine.

Materials and methods. 300 Webster female albino mice (18 to 25 g), 450 Wistar female albino rats (100 to 125 g), and 15 adult mongrel dogs of both sexes were used. Presence or absence of central depressant effect in mice and rats was determined by observation of running movements, restlessness, convulsions, incoordination, and loss of righting reflex. In dogs the appearance of miosis, bradycardia, emesis, and convulsions were noted. The prolongation of hexobarbital hypnosis in mice and dogs was observed by noting the time of loss of the righting reflex; control animals were treated with saline before hexobarbital administration. Effects of drugs on pentylenetetrazole induced convulsions in rats were determined by a previously described technic (2). Anticonvulsant effects were studied against 2 dose levels of pentylenetetrazole, 75 mg/kg (LD_{75}) and 100 mg/kg (LD_{100}), both doses producing convulsions in 100% of the untreated rats. The incidence of convulsions and death in 10 rats receiving the anticonvulsant ½ hour before pentylenetetrazole was compared with the incidence in 20 control rats which received pentylenetetrazole alone.

Results. Effects on C.N.S. Doses of MEM in mice up to 480 mg/kg subcutaneously (s.c.) and 900 mg/kg orally did not produce depressant or stimulant effects, loss of righting reflex, or catatonus of the tail. The incidence of convulsions was very low even at the toxic oral dose of 640 mg/kg. On the other hand, codeine in mice at doses from 90 to 180 mg/kg intraperitoneally (i.p.) produced restlessness, tail catatonus, and convulsions. Ketobemidone and dl-Methadone, both at 6 mg/kg i.p., induced a very moderate restlessness in mice, and Miltown induced paralysis and loss of righting reflex at 200 to 400 mg kg s.c. In rats MEM produced no effects until a dose of 300 mg/kg s.c. was given, which produced relaxation and flaccidity. In dogs no effect was observed up to 150 mg/kg s.c., but 300 mg/kg induced ataxia, general depression, and tran-

TABLE I. Prolongation of Hexobarbital Sodium Hypnosis. (100 mg/kg i.p.) by previous administration of MEM, codeine, and chlorpromazine.

Exp. No.	Drug	Dose	Route of admin.	Time before hexobarbital (min.)	Mean duration of hypnosis (min.) ± S.E.	t	P
I	Saline	.1 cc/10 g	S.C.	30	19 ± 2.36	—	—
	MEM	75 mg/kg	"	"	26 ± 3.50	1.67	.1
	"	150 "	"	"	53 ± 10.15	3.27	<.01
	Codeine (ph^{1e})	15 "	"	"	32 ± 5.40	2.20	.04
II	Saline	.1 cc/10 g	"	"	31 ± 2.61	—	—
	MEM	300 mg/kg	"	60	61 ± 5.63	4.84	<.01
	"	300 "	Oral	"	43 ± 6.01	1.84	.09
	Codeine (ph^{1e})	30 "	S.C.	"	61 ± 7.64	3.72	<.01
III	Saline	.4 cc/10 g	Oral	"	40 ± 4.86	—	—
	MEM	300 mg/kg	"	30	55 ± 6.83	1.78	.1
	"	600 "	"	"	65 ± 8.32	2.60	.02
	Codeine (ph^{1e})	600 "	"	60	60 ± 8.83	1.98	.06
IV	Saline	.1 cc/10 g	S.C.	30	26 ± 3.54	—	—
	Chlorpromazine	.5 mg/kg	"	"	31 ± 4.48	.88	.42
	"	1 "	"	"	41 ± 4.40	2.68	.02
	"	5 "	"	"	88 ± 7.45	7.96	<.01

10 mice used for each experiment. S.E. = Stand. error of mean.

sient bradycardia. Codeine in dogs induced excitement and convulsions at 75 mg/kg s.c. and was fatal at 150 mg/kg.

Prolongation of hexobarbital sleep. The results of these experiments in mice are shown in Table I. It can be seen that doses of MEM which were not depressant in themselves significantly increased the hypnotic effect of hexobarbital sodium. The extent of the effect was related to magnitude of dose. The hypnotic effect of MEM was no greater than that of codeine or chlorpromazine. A prolongation of hexobarbital hypnosis was also observed in dogs with MEM 30 mg/kg s.c. and with codeine 7.5 mg/kg s.c. given ½ hour before hexobarbital 50 mg/kg intravenously (i.v.).

Effect on pentylenetetrazole induced convulsions. Fig. 1 shows that MEM was strikingly effective in protecting rats from convulsions induced by 75 mg/kg pentylene tetrazole; the ED_{50} for MEM was 200 mg/kg. A good anticonvulsant effect was also noted with Miltown 30 to 120 mg/kg, confirming Berger's data(3) with mice. On the other hand, morphine, dl-Methadone, l-Methadone, ketobemidone, codeine, l-Isomethadone, and Meperidine were all ineffective, although a trend towards protection was seen with small doses of the latter 3 drugs. Morphine also had a fair anticonvulsant action at the smaller dose of 3 mg/kg. MEM effectively antagonized the convulsions induced by 100 mg/kg pentylenetetrazole, the ED_{50} in this case being 450 mg/kg.

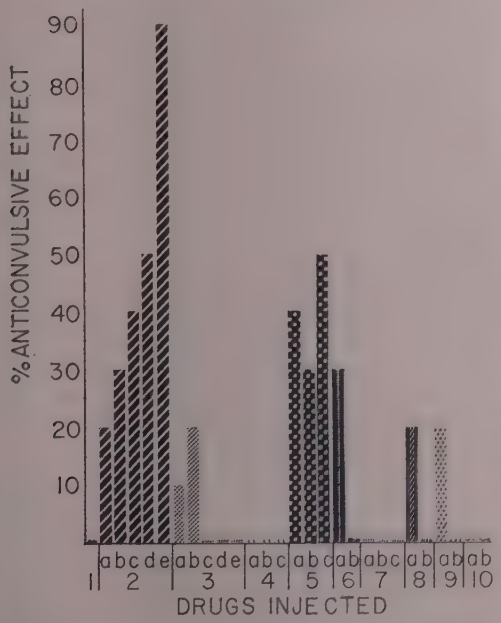


FIG. 1. Effect of various drugs (mg/kg) in antagonizing convulsions in rats induced by pentylenetetrazole 75 mg/kg i.p.: (1) Controls; (2) MEM: a. 50, b. 150, c. 200, d. 300, e. 450; (3) codeine phosphate: a. 5, b. 10, c. 15, d. 30, e. 315; (4) dl-Methadone: a. 3, b. 10, c. 30; (5) Miltown: a. 30, b. 60, c. 120; (6) morphine sulfate: a. 3, b. 30; (7) ketobemidone: a. 1.5, b. 5, c. 15; (8) Meperidine HCl: a. 30, b. 300; (9) l-Isomethadone: a. 3, b. 30; (10) l-Methadone: a. 1.5, b. 15.

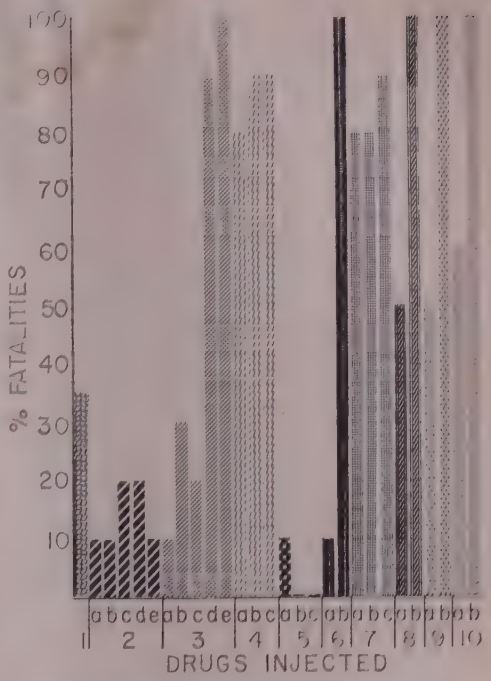


FIG. 2. Effect of various drugs (mg/kg) in rats on mortalities induced by pentylenetetrazole 75 mg/kg i.p.: (1) Controls; (2) MEM: a. 50, b. 150, c. 200, d. 300, e. 450; (3) codeine phosphate: a. 5, b. 10, c. 15, d. 30, e. 315; (4) dl-Methadone: a. 3, b. 10, c. 30; (5) Miltown: a. 30, b. 60, c. 120; (6) morphine sulfate: a. 3, b. 30; (7) ketobemidone: a. 1.5, b. 5, c. 15; (8) Meperidine HCl: a. 30, b. 300; (9) l-Isomethadone: a. 3, b. 30; (10) l-Methadone: a. 1.5, b. 15.

Effect on pentylenetetrazole toxicity. MEM 25 to 450 mg/kg s.c. in rats was very effective in preventing the lethal effects of LD_{95}

and LD_{100} pentylenetetrazole, as shown in Fig. 2. The effectiveness of Miltown in this respect again confirms Berger's data with mice(3). However, methadone isomers and high doses of morphine and codeine significantly potentiated pentylenetetrazole toxicity, while the low doses of the latter 2 drugs were protective.

Discussion. The results of these experiments show clearly that MEM, unlike morphine, codeine, and methadone isomers, is devoid of a central excitatory action in non-toxic doses in the 3 species studied. It is for this reason that MEM significantly protects against pentylenetetrazole convulsions and fatalities, while morphine, codeine, and the methadones potentiate these effects. However, we have found that smaller doses of these latter compounds, in contradiction to the data of Hazelton and Koppanyi(4,5), are also protective against the effects of pentylenetetrazole; these doses probably produce only C.N.S. depression without a simultaneous stimulatory action.

Summary. (1) In non-toxic doses MEM shows no C.N.S. excitatory effects in mice, rats, and dogs. (2) In doses which do not induce loss of righting reflex, MEM prolongs hexobarbital hypnosis. (3) MEM, unlike morphine, codeine, and some methadone isomers, significantly blocks the convulsant and toxic effects of pentylenetetrazole.

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Frequency of t^w Alleles in a Confined Population of Wild House Mice.* (22460)

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The T locus in mice represents a complex locus in which 11 reported alleles, many of them proven to be different from others, have been derived from laboratory stocks. Many populations of wild mice have also been shown to contain recessive mutants at the T locus. Some of these wild alleles have been shown to be different from those found in laboratory stocks(1). The dominant allele, T, is homozygous lethal and when present in heterozygous condition, $T/+$, with the normal allele causes a variable shortening of the tail (Brachury = short tail). In addition to T there is a series of recessive alleles, t^n, each of which when present with T (as T/t^n) produces the tailless phenotype. The recessive alleles, t^n, may be homozygous lethal or viable. When viable, the males of the genotype, t^n/t^n, are usually sterile. Viable female mice of the genotype, t^n/t^n, are usually fertile. It is relatively simple to discover whether a mouse has the genotype $+/t^n$ or $+/+$. The animal in question is mated to a Brachy ($T/+$). If the animal in question is $+/+$ only two types of offspring are obtained: normal tailed and Brachy, in approximately equal numbers. Should the mouse in question, however, be $+/t^n$, then 3 classes of offspring are found; namely, normal tailed, Brachy and tailless. This latter group has the genotype, T/t^n. Provided that sufficient numbers of offspring are obtained from a test animal, it can be classified as $+/+$ or $+/t^n$. In males the procedure is greatly aided by the phe-

* This work was carried out at the Nevis Biological Station, Irvington-on-Hudson, New York, under contract AT(30-1)-1804, U. S. Atomic Energy Commission.

nomenon of "segregation ratio abnormality" in which $+/t^n$ male mice usually produce many more t^n sperm than $+$ sperm. As a result many male mice that are $+/t^n$ have tailless offspring in their first litters(2). In practice, new t alleles have been numbered in the order of their discovery. It may later be found that some of the numbered t alleles are indistinguishable from each other and the higher numbered line is then discarded. For such t alleles as are discovered in wild populations of mice, the superscript "w" is added to indicate their origin, hence t^{w1}, t^{w2}, etc.

The present paper is concerned chiefly with the problem of determining the frequency of heterozygotes in a population known to possess t^w. This population is descended from wild mice caught in the suburbs of New York and Philadelphia in 1943-44 and since maintained as a small (125-250 individuals) laboratory population by Dr. Howard A. Schneider of the Rockefeller Institute for Medical Research, New York. It has undergone moderate in-breeding (usually by half-sibling matings, with avoidance of full sibling mating) (3). Dr. Schneider has kindly supplied the samples tested.

Previous studies. The first sample (R – 1) tested (January 1952) consisted of 10 males of which 8 produced test offspring. Of these, 5 proved to be $+/t^w$. Two different alleles were found: t^{w1}, homozygous lethal; t^{w2}, homozygous viable. Males t^{w2}/t^{w2} proved to be sterile; t^{w2}/t^{w2} females were fertile(2). A second sample (R – 2) of 6 males and 21 females was tested beginning August 1952. Four males and 11 females gave test progenies. Of these 2 males and 4 females proved to be $+/t^w$ and again both alleles t^{w1} and t^{w2} were present.

Present study. A further sample (R – 3) from this confined population consisting of 68 females and 70 males was obtained in June 1955. These were mated with Brachys ($T/+$) and tested individually. Thirteen females (19%) and 26 males (37%) gave no litters after 4 months of testing by fertile mates, a sterility rate of 28% compared with 33% previously found(4).

Of the fertile animals, 22 out of 44 males (50%) and 36 out of 55 females (65%) produced at least 2 tailless offspring each (usually more) and were diagnosed as $+/t^w$. There was thus found a minimum of 58 heterozygotes out of 99 tested (59%). The results are shown in Table I.

TABLE I. Results of Testing Wild Type Mice by Brachy ($T/+$) Mice.

	Offspring			
	Normal	Brachy	Tailless	
Genotype	$+/t^w$	$T/+$	T/t^{w2}	Total
22 $+/t^{w2}$ ♂	107	13	80	200
22 $+/+$ ♂	252	237	—	489
26 sterile ♂	—	—	—	—
36 $\frac{+/t^{w2}}{t^{w2}\ t^{w2}}$ ♀	150	64	101	315
19 $+/+$ ♀	131	104	—	235
13 sterile ♀	—	—	—	—

Some of the tailless offspring were tested for the presence of t^{w1} and t^{w2}. Of 13 $+/t^w$ animals whose descendants were tested all proved to contain t^{w2} only. In the 2 previous samples, we estimated the relative frequencies of t^w alleles as 25% t^{w1} and 75% t^{w2}. Assuming such frequencies the probability of getting 13 t^{w2} out of 13 tested is less than 3%. Hence t^{w1} had either disappeared or was very rare in the third sample.

The apparent disappearance of t^{w1} from the population may have been either due to a superiority of t^{w2} or to random breeding. This population is reduced each summer from about 250 to approximately 125 animals. It is possible that in one of the reductions between 1952 and 1955 inadvertently only t^{w2}-containing mice were retained. Should this be the case, it would constitute an actual example of *random genetic drift* resulting in the elimination of a gene from a population.

In Table I, the ratio of tailless to tailless plus Brachy offspring, from the cross $+/t^w$ x $T/+$, expressed the proportion of t^w gametes in the $+t^w$ parent. For males the proportion is .86, indicating a preponderance of t^w sperm, such as is usually found in male heterozygotes(5). However, in the case of t^w-containing females the proportion is .61, which seems to indicate an excess of t^w eggs. No definite statement about a possible segre-

gation ratio in females of this population can be made as it was not possible to test each mutant carrying female as to whether she was homozygous or heterozygous for t^{w2}. Much of the excess of t^w eggs may be due to females, homozygous for t^{w2}. Such females are both viable and fertile and when mated to Brachy males can give only normal tailed and tailless offspring.

Discussion. It is interesting to contrast the frequency of t^w-containing individuals in this confined population (59%) with those found in newly caught wild animals. This latter frequency has been estimated as a minimum of 32%(1). Confined populations and wild populations of mice are undoubtedly subject to different selection pressures. It is possible that conditions in a confined population favor t^w-containing individuals more than do conditions in wild populations. Experiments have shown that, in this confined population, male mice heterozygous for a lethal ($+/t^{w1}$) have a higher net relative fertility than $+/+$ males(4).

The lack of adequate samples in previous studies makes it impossible to state whether 59% represents the equilibrium frequency of t^{w2} in this population. The population may still be in a state of flux and t^{w2} may eventually either be eliminated or reach fixation. Prout has considered the possible fates of t^w alleles in a population(6).

Two observations which may be casually related are the relatively high frequency of males that gave no litters and the relatively low frequency of $+/t^w$ males, as compared with females in this population. These phenomena may be due to the peculiar characteristics of t^{w2} homozygotes which, as males, are viable but sterile. Females, homozygous for t^{w2}, are both viable and fertile.

Summary. 1. A sample (R – 3) from a confined population of mice, consisting of 70 males and 68 females, was tested for the presence of t^w alleles. This population is descended from animals caught in the wild. 2. Of 44 tested males, 50% were $+/t^w$; while of 55 tested females, 65% contained t^w. This population has an overall-frequency of 59% t^w containing fertile individuals. The present generation of this population seems to contain only t^{w2}, a viable allele, and $+$. Previously tested generations (R – 1 and R – 2) had contained both t^{w2} and t^{w1}. The latter is a lethal allele. 3. The $+/t^{w2}$ males of this population transmitted t^{w2} gametes to their offspring in excessive numbers, a phenomenon known from other studies and referred to as "segregation ratio abnormality." 4. Discussed is the overall high frequency of t^w-containing individuals found in this population as compared with animals caught in the wild. Also discussed is the disparity between males and females with respect to frequencies of sterility and t^w-containing individuals.

The authors take pleasure in acknowledging the cooperation of Mr. Robert E. Stephenson, superintendent of Nevis, and the assistance of David Bruck, Andrew B. Beasley and Frank Burnett, whose help was indispensable for the successful completion of this project.

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Effect of Metabolites on Accumulation of Citrate in Fluorocitrate-poisoned Rats.* (22461)

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The effect of metabolites of the Krebs cycle on the accumulation of citrate in fluoroacetate-poisoned rats has been reported(1). C_4 metabolites like α-ketoglutarate, succinate, malate and oxalacetate increase the accumulation of citrate in the heart, and to a lesser extent in the kidney, over and above that observed after fluoroacetate alone. The C_2 fragments like acetate and ethanol diminish the accumulation of citrate, possibly by competing with fluoroacetate for coenzyme A, on the assumption that the real poison is fluorocitrate(2). Fluoroacetate can thus be used as a tool in studying the intermediary metabolism of certain substances. For instance, we have found that pyruvate and butyrate behave in the heart like C_4 fragments of the Krebs cycle. The change of pyruvate into oxalacetate may be explained by CO_2-fixation, but the behavior of butyrate cannot be explained on the basis of current concepts of intermediary metabolism. Propanol and propionate behave like C_2 fragments and are the most effective in inhibiting citrate accumulation in fluoroacetate-poisoned rats. The lack of accumulation of citrate in the liver of fluoroacetate-poisoned rats was explained by the assumption that fluoroacetate would be diluted in this chief acetylating organ, in a big pool of C_2 fragments.

If fluoroacetate blocks the Krebs cycle by virtue of a "lethal synthesis" of fluorocitrate (2), an inhibitor of aconitase, it follows that an equimolar dose of fluorocitrate should be more effective than fluoroacetate, since only part of the fluoroacetate would be synthesized into fluorocitrate because of dilution of fluoroacetate in the acetate pool. The results to be reported herein were not as predicted and they show great differences in different organs. An attempt will be made to explain these variations.

* This study has been supported by a grant from the American Heart Assn.

Methods. Adult male albino rats, 4 to 7 months old, were used. Although these were of a different strain than those used in the previous study(1) yet the "resting" tissue citrate values were the same in both strains. Synthetic fluorocitric acid(3), 12.7 mg per kg, was neutralized and injected intraperitoneally 30 minutes after the subcutaneous injection of the metabolite. This quantity of fluorocitrate is double the molar dose of fluoroacetate used previously since Peters(4) has shown that synthetic fluorocitrate is half as effective as the "enzymatic" fluorocitrate in inhibiting the aconitase of kidney particles. The rest of the experimental technic has been described previously(1).

Results. In 6 control rats without poison the average citrate content of the tissues in μg per gram was as follows: Heart, 23 $\pm$ 2.5; Kidney, 45 $\pm$ 5.5; Liver, 46 $\pm$ 3.7; Brain, 41 $\pm$ 1.8; Plasma, 30 $\pm$ 2. The results with fluorocitrate are summarized in Table I.

Heart. Fluorocitrate increases the heart citrate much less than fluoroacetate, 150 μg compared to ca. 1000 μg. Part of the 150 μg of citrate are due to plasma and extracellular fluid in the heart but this does not account for the whole increase since the plasma citrate content of these rats is only 276 $\pm$ 20 μg per ml. It appears then that the heart is very slightly permeable to fluorocitrate. The increase in citrate content after butyrate is not surprising since unpoisoned hearts react likewise(1). Starvation increases the citrate level but this is observed also in nonpoisoned animals, (unpublished experiments).

Brain. There is little accumulation of citrate in the brain of fluorocitrate-poisoned rats. The small increase may be accounted for by the plasma and extracellular fluid content of the brain. However, the magnitude of these figures is unknown to us. Furthermore

TABLE I. Citrate Accumulation in Tissues of Fluorocitrate-Treated Rats after Injection of Various Metabolites. Mean values ± S.E. are given in μg/g wet tissue. Unless otherwise stated, 2.25 millimoles of metabolite were inj./100 g body wt.

Metabolite	No. of animals	Heart	Kidney	Liver	Brain
Saline controls	27	148 ± 13	1760 ± 56	1790 ± 19	77 ± 4
Succinate	10	71 ± 8	731 ± 55	1040 ± 106	68 ± 3
Malate	9	147 ± 14	1470 ± 74	1095 ± 74	117 ± 16
Acetate*	10	187 ± 18	1780 ± 140	1317 ± 86	105 ± 10
Ethanol†	10	134 ± 17	1797 ± 81	527 ± 85	66 ± 4
Propanol	10	98 ± 15	1638 ± 66	1058 ± 81	84 ± 7
Propionate	3	132 ± 21	2027 ± 129	1194 ± 69	96 ± 20
Acetone†	10	166 ± 20	1830 ± 87	1507 ± 56	79 ± 6
Butyrate†	5	497 ± 16	2720 ± 195	1370 ± 156	74 ± 8
Starvation	10	213 ± 12	1665 ± 40	316 ± 42	80 ± 7

* 3.1 millimoles/100 g body wt.
† 4.5 *Idem*

Peters *et al.*(4) obtained values of ca. 250 μg per gram after using 4 times the dose employed here. It would seem then, that the brain is also only slightly permeable to fluorocitrate.

Kidney. There is more accumulation of citrate in the kidney with fluorocitrate than with fluoroacetate. Here again, the increase in citrate after butyrate reminds one of the condition obtained in the nonpoisoned kidney. The C_4 fragments cause a decrease in accumulated citrate.

Liver. Here, high values, ca. 1800 μg, are obtained with fluorocitrate, whereas with fluoroacetate there is a slight or no increase, (4,5). The effect of metabolites on the liver citrate were not predictable according to our experience with the heart and kidney of fluoroacetate-poisoned animals, for here both the C_4 and C_2 fragments of the Krebs cycle inhibited the citrate accumulation in the liver, ethanol being most active. A possible explanation for these findings will be given below. Starvation for 48 hours has a profound influence on citrate accumulation reducing it to one-sixth the value obtained in fed animals.

Discussion. The fact that there is no significant accumulation of citrate in the heart of fluorocitrate-poisoned rats is most probably attributable to the lack of diffusibility of fluorocitrate and does not suggest that fluoroacetate and fluorocitrate act by two different mechanisms. In unpublished work from this laboratory on the effect of fluoroacetate on the dog heart-lung preparation it was shown that the heart citrate can increase 20-fold without a trace of it diffusing into the serum. The same considerations may explain the insignificant rise in brain citrate after fluorocitrate.

C_4 intermediates of the Krebs cycle cause a decrease of citrate accumulation in the liver and kidney of fluorocitrate-poisoned animals instead of an increase as with fluoroacetate. We venture the following explanation for the difference. Owing to the poor diffusibility of fluorocitrate the more diffusible C_4 fragments, injected 30 minutes before fluorocitrate, may have increased the citrate content to such an extent as to protect aconitase against slowly diffusible fluorocitrate. The action of the C_2 fragments was also not predictable. We did not expect a decrease of accumulated citrate as in fluoroacetate poisoning, since here there is no competition with fluoroacetate for coenzyme A. However, a decrease did take place. Experiments are in progress to elucidate this "anomalous" behavior of C_2 fragments in fluorocitrate poisoning as well as that of butyrate in fluoroacetate poisoning.

Starvation reduces citrate accumulation in fluorocitrate-poisoned rats to one-sixth the value found in fed rats. This may suggest that in starvation, the Krebs cycle operates at a reduced rate.

Summary. 1) Fluorocitrate causes much less accumulation of citrate in the heart than does fluoroacetate. This is most likely due to relative impermeability of the heart to fluorocitrate. The same appears to apply to the brain. 2) C_2 as well as C_4 fragments of the Krebs cycle decrease the accumulation of citrate in the liver of fluorocitrate-poisoned ani-

mals. 3) Forty-eight hours' starvation reduces the citrate accumulation in the liver of fluorocitrate-poisoned rats to one-sixth the value obtained in fed animals.

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Effects of X-Irradiation on Androgenic Response of Seminal Vesicles of the Castrate Rat.* (22462)

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In investigating cytological effects of testosterone propionate on secretory epithelium of rat seminal vesicles, Cavazos and Melampy (1) observed no mitoses in epithelium of castrates or 24- and 36-hour hormone-treated animals. However, mitotic activity of 7.5% was noted 60 hours after initial hormone treatment. Following this maximum there was a sharp decline in mitoses and in animals treated with male hormone for 20 days a value of 0.1% was observed. Colchicine was used in these experiments.

Regressed seminal vesicles of the castrate rat provide useful test tissue for such investigation because cells of secretory epithelium are in the resting stage and can be reactivated with androgen. Observations are reported on effects of various levels of X-irradiation on 60-hour response of seminal vesicles to testosterone propionate (T.P.).† Data were obtained on mitotic index and cell height of secretory epithelium as well as total nitrogen content of the organ.

Materials and methods. 159 rats of Holtzman strain were used and fed Purina Laboratory Chow. At time of castration average animal weights ranged from 266 to 312 g and seminal vesicles were allowed to regress 20 days before X-irradiation and/or hormone treatment. The initial dose of androgen was administered at time of irradiation. Hormone-treated castrates received daily 500 μg of T.P. in oil injected subcutaneously, total amount 1500 μg. Control groups received 0.05 ml of oil daily and no irradiation. Experimental animals were sacrificed 60 hours following treatment. Animals were exposed to X-rays from General Electric Maxitron operated at 130 kvp and 10 ma with 0.25 mm Al filtration and at 20 cm target distance. Nembutal anesthesia was employed during irradiation (2.5 mg/100 g body wt). Animals were shielded with lead except in pelvic area. Individuals used to furnish data on mitotic index and cell height of secretory epithelium were injected subcutaneously with 0.1 mg of colchicine per 100 g body weight 6 hours prior to killing according to Burkhart(2). Percentages of mitotically dividing nuclei, mitotic index, and cell heights were determined by procedure described earlier(1). Total nitrogen data were obtained by a micro-Kjeldahl method.

Results. The results of this investigation are summarized in Tables I and II. Data are presented as means with their standard errors.

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† Peranderen, Ciba Pharmaceutical Products, Summit, N. J.

TABLE I. Effects of X-Irradiation on Body Weight and Androgenic Response of Secretory Epithelium of Seminal Vesicles of Castrate Rat.

Animals per group	Treatment Irradiation, r	Treatment Hormone daily, μg	Body wt at castration, g	Body wt at irradiation, g	Body wt 60 hr after treatment, g	Seminal vesicles wt, mg	Mitotic index, %	Cell height, μ
4	0	0	275 ± 8*		325 ± 9*	96 ± 8*	0	8 ± .4*
7	0	500	294 ± 9		344 ±10	219 ± 9	8 ± 1*	17 ± .3
6	80	"	288 ±10	319 ±11*	320 ±10	207 ±13	14 ± 1	19 ± .3
5	160	"	296 ± 8	325 ±10	325 ± 9	218 ± 7	20 ± 1	19 ± .6
5	160	0	304 ± 7	346 ± 8	330 ± 8	95 ± 5	0	7 ± .3
7	320	500	274 ± 6	305 ± 7	306 ± 8	214 ±11	17 ± 1	18 ± .4
7	640	"	276 ± 5	315 ± 5	303 ± 5	205 ±19	15 ± 1	18 ± .0
5	1000	"	281 ± 6	318 ± 3	285 ± 4	185 ± 5	5 ± 1	17 ± .3
7	1500	"	291 ± 9	327 ± 7	309 ± 7	181 ± 7	4 ± 1	18 ± .4
7	2000	"	261 ± 5	314 ± 5	293 ± 6	184 ± 7	3 ± 1	18 ± .3
8	3000	"	308 ± 4	337 ± 3	311 ± 4	198 ± 5	1 ± 0	17 ± .7

* Mean ± S.E.

Discussion. Mitoses were not observed in regressed seminal vesicles of control animals but a mitotic index of 8% was obtained in animals 60 hours after initial dose of hormone and no X-irradiation. In androgen-treated groups receiving 80 and 160 r this value increased to 14 and 20%. Mitotic activity was absent in secretory epithelium of animals which received 160 r without androgen. A mitotic index of 10 ± 0.5% was obtained in a group of 5 rats irradiated with 160 r one hour before colchicine treatment (53 hours after initial hormone injection). Bloom(3) indicated there is no experimental evidence that X-irradiation induces cell division. In hormone-treated castrates it is possible that irradiation at levels between 80 and 640 r caused a delay in mitotic response of secretory epithelium and as a result a greater mitotic index was observed at 60 hours. Animals receiving 3000 r showed 1% of epithelial nuclei dividing indicating destructive action of higher dosage. Fleischmann and Nimaroff (4) have demonstrated X-irradiation of accessory sex organs of castrate male rats and immediate subsequent androgen treatment resulted in cellular hypertrophy with delayed mitoses. However, if an interval of 5 days was allowed between irradiation and administration of male hormone this delaying effect was lost. Data suggesting recovery from irradiation effects were also obtained. An average mitotic index of 2% was found in a group of 8 animals irradiated with 3000 r 48 hours prior to initial hormone injection, whereas no mitoses were observed in a similar group of 8 rats given same dosage 48 hours following androgen.

Data presented in Table I indicate that under conditions of these experiments X-irradiation from 80 to 3000 r had no significant effect on cell height in animals receiving male-sex hormone. The weight of seminal vesicles in hormone-treated animals without irradiation (Table II) was more than double the weight of controls. A decrease was noted in weights of seminal vesicles of animals receiving 500 μg of hormone daily and increasing amounts of X-irradiation (Table II). There was a 3-fold increase in nitrogen content of seminal vesicles following androgen administration without irradiation when compared to controls. However, at 3000 r the nitrogen content was more than twice that of controls which received neither irradiation nor hormone. The decreasing amounts of nitrogen per gland as shown in Table II are the result of a fall in organ weight rather than a significant change in percentage of nitrogen.

Table II indicates that irradiation at highest levels (1500, 2000, and 3000 r) reduced seminal weight by approximately 30% when compared with hormone-treated animals. The same levels of irradiation resulted in lowest observed mitotic indices (Table I). This finding is in agreement with observations of Fleischmann and Brackin(5) that growth induced by testosterone propionate in accessory sex organs of castrate male rats is only partially inhibited by irradiation with X-rays.

Summary. Mature male rats were cas-

TABLE II. Effects of X-Irradiation and Testosterone Propionate on Body and Seminal Vesicle Weights and Nitrogen Content of Seminal Vesicles of Castrate Rat.

Animals per group	Treatment Irradiation, r	Treatment Hormone daily, μg	Body wt at castration, g	Body wt at irradiation, g	Body wt 60 hr after treatment, g	Seminal vesicles wt, mg	N/gland, mg
13	0	0	305 ± 5*		347 ± 6*	107 ± 4*	1.8 ± .1*
13	0	500	304 ± 6		344 ± 7	233 ± 8	5.5 ± .1
6	80	"	293 ± 9	337 ± 7*	337 ± 6	207 ± 7	4.9 ± .1
6	160	"	294 ± 3	351 ± 6	347 ± 7	222 ±10	5.1 ± .2
4	320	"	270 ±10	326 ±11	324 ±12	209 ± 3	5.2 ± .1
8	640	"	303 ± 3	334 ± 7	318 ± 5	193 ± 9	4.7 ± .2
5	1000	"	312 ± 4	342 ± 5	320 ± 4	197 ± 4	4.6 ± .1
5	1500	"	296 ± 1	348 ± 3	312 ± 3	180 ± 5	3.6 ± .3
5	2000	"	285 ± 7	334 ± 7	298 ± 7	159 ± 8	3.9 ± .4
5	3000	"	284 ± 3	337 ± 5	301 ± 3	166 ± 5	4.0 ± .2

* Mean ± S.E.

trated and accessory organs regressed 20 days before X-irradiation and/or hormone treatment. Androgen-treated castrates received daily 500 μg of testosterone propionate in oil, total amount 1500 μg. Animals were sacrificed 60 hours following initial treatment. The mitotic index with hormone was 8%, whereas following androgen and irradiation at 80, 160, 320 and 640 r it was 14, 20, 17 and 15% respectively. At levels of 1000, 1500, 2000, and 3000 r these values were 5, 4, 3 and 1%. These data indicate inhibition of mitotic activity at levels of 1000 r and above. It is suggested that irradiation between 80 and 640 r caused a delay in mitotic response of secretory epithelium and as a result the mitotic index was greater than in those which received androgen alone. X-irradiation had no significant effect on cell height. There was a decrease in seminal vesicle weights of animals treated with hormone and increasing amounts of irradiation.

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Effect of Malonate on Selected Body Defenses.* (22463)

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Previous work from this laboratory(2-7) has shown that repeated injections of sublethal doses of malonate reduce survival time of mice infected with *Salmonella typhimurium* from 64-84 hours to 8-24 hours. Survival time is also reduced by injections of fluoroacetate, arsenite, citrate, and succinate. Bacteria multiply more rapidly in malonate treated mice than in saline treated mice as revealed by total viable pathogen counts in homogenates prepared from mouse carcasses (7). Survival time seems dependent, therefore, upon time required for microorganisms to reach a fairly constant lethal number. The greater rate at which this lethal number of pathogens is reached in mice, given malonate, may be attributed to one or both of the following conditions: (a) decreased rate of destruction due to impairment of animal's body defenses or (b) more rapid rate of reproduction due to more favorable nutritive environ-

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ment. Whether the increased susceptibility associated with injections of malonate can be correlated with impairment of selected phases of the defense systems is the subject of the present report.

Materials and methods. Survival tests. Rats weighing between 50-65 g were infected intraperitoneally with 0.5 ml of a saline suspension of *S. typhimurium* containing approximately 45 x 10^6 cells. Bacteria were cultured 15 hours in brain-heart infusion broth (Difco). Three groups of rats were employed. Two groups were infected and 30 minutes later the first of 8 hourly injections of either 0.5 mg malonate per g of rat contained in approximately 0.5 ml of sterile saline or comparable volume of saline alone was administered. The third group, not infected, was given injections of malonate and served as toxicity control. *Phagocytic measurements.* Human blood was defibrinated and added to an equal volume of saline. Rat blood, obtained by cardiac puncture with the animal under light ether anesthesia; was added to equal volume of saline containing 0.5 mg heparin per ml. Heparin at this concentration has no observable effect on phagocytic activity of neutrophiles(1). Malonate was introduced into the test systems by adding to 0.5 ml blood an equal volume of disodium malonate (Eastman) dissolved in sterile saline or by administering a series of 8 hourly injections of 0.5 mg of malonate per g body weight. The animals were bled 15 minutes after the last injection. The method of Cottingham and Mills(8) was followed for phagocytic tests. One ml blood-saline mixture was brought to 37°C by gentle agitation in water bath. Agitation was continued for 15 minutes after addition to each tube of 0.2 ml suspension of *Staphylococcus aureus,* density adjusted in a Coleman Spectrophotometer, Model 11, to give a light transmittance of 70% at wave length of 650 mmu. Smears were made on 2 cover slips and stained with Wright and Giemsa stains. Phagocytic activity is expressed as percent of neutrophiles showing engulfment of one or more bacteria determined by microscopic inspection of 100 to 200 cells. All slides were evaluated without knowledge of groups to which they belonged. *Serum bactericidal activity.* Blood of 3 or 4 rats was defibrinated and pooled. Following centrifugation, 3 ml of serum was brought to temperature in a 37°C incubator, during which time a suspension of *S. typhimurium,* cultured for 15 hours in brain-heart infusion broth (Difco), was prepared by serial dilutions with sterile saline. To the serum, 0.1 ml of the suspension was added. At hourly intervals thereafter, 0.1 ml of serum-bacteria mixture was pipetted off, diluted with saline, and 0.1 ml cultured in triplicate on SS agar. Number of colonies times dilution factor gave the number of viable bacteria per ml of mixture. The effect of malonate on bactericidal activity of serum was tested by bleeding rats one-half hour after administration of the second of 2 hourly injections of 0.5 mg malonate per g body weight. For experiments requiring addition of zymosan, this compound was prepared, with some modifications, from fresh yeast according to Pillemer and Ecker(9). Zymosan was suspended in saline for addition to serum according to Pillemer, *et al.*(10). Final concentration was 3 mg zymosan per 1 ml serum. Zymosan and serum were incubated at 37°C for one hour prior to addition of bacteria. *General.* Male and female white rats (Carworth Farms) were used. The rats were fed Purina dog chow checkers and water was available at all times.

Results. Survival time of infected rats given malonate. In 2 experiments survival time of rats infected intraperitoneally with *S. typhimurium* and then administered a series of 8 hourly injections of 0.5 mg malonate per g body weight was significantly less than that of control rats similarly infected and given injections of saline. The average survival time of the group given malonate was 48 hours compared with 114 hours for the controls ($P < 0.05$ according to rank order test(12)) in the first experiment, while in the second, values were respectively 8.5 hours and 118 hours ($P < 0.001$). None of the uninfected rats given the same number of injections of malonate, died. These results compare favorably with those previously reported for mice(2,4).

Phagocytic measurements with human neu-

TABLE I. Phagocytic Activity of Human Neutrophiles Expressed as Percent of Cells Ingesting Bacteria. All values in each row are averages of 2 separate determinations made on the same sample of blood.

A .09% saline			B .30% saline			C .90% saline			D Control		
1	2	3	1	2	3	1	2	3	1	2	3
15 mg*	5 mg*	1 mg*	15 mg*	5 mg*	1 mg*	15 mg*	5 mg*	1 mg*	sal.	sal.	sal.
%											
62	66	—	66	78	89	62	65	88	89	97	79
68	83	87	70	75	87	—	71	88	84	84	82
66	71	86	60	73	92	57	72	88	86	92	91
56	72	83	67	88	85	63	89	84	75	83	85
Avg 63	73	85	66	79	88	61	74	87	84	89	84

* Concentration of malonate/ml blood-saline mixture.

trophiles. The percent of active phagocytes obtained with human neutrophiles is listed in Table I. Sections A, B, and C represent values obtained with 3 different concentrations of saline to make the final solution containing malonate approximately isotonic with blood. Thus 0.09% saline and 15 mg of malonate, 0.30% saline and 5 mg malonate, and 0.9% saline and 1 mg malonate have similar calculated tonicities. It is evident, however, that no significant difference in phagocytic activity can be attributed to osmotic effects.

The average phagocytic activities determined in presence of 15 mg malonate per 1 ml blood-saline mixture and in 3 concentrations of saline, are 63%, 66%, and 61%. The corresponding average for phagocytic activity, in the absence of malonate, is 84% (column D_1). Data presented in columns A_2, B_2, and C_2 were obtained from phagocytic tests employing 5 mg malonate. Control values are shown in column D_2. Average percentages of phagocytic activity of experimental groups are 73%, 79%, and 74%, while control values average 89%. In the presence of 1 mg malonate (columns A_3, B_3, and C_3) average percentages are 85%, 88%, and 87%. Control values average 84%.

The data of Table I were subjected to statistical analysis, using the rank order test. Comparisons were made between all values obtained with each concentration of malonate regardless of strength of saline in which it was dissolved. All differences are highly significant (P values of 0.01 or less) except for data obtained with 1 mg of malonate and with controls, where the P value is greater than 0.05.

TABLE II. Phagocytic Activity of Rat Blood Neutrophiles Expressed as Percent of Cells Showing Ingestion of Bacteria. Each value was obtained with blood from a different rat.

A		B	
1	2	1	2
1 mg malonate *in vitro*	Saline *in vitro*	8 inj. of 0.5 mg malonate per g body wt	8 inj. of saline
%			
85	84	83	89
73	88	90	—
84	—	86	—
77	91	84	95
84	—	87	—
92	81	95	—
		81	69
		84	79
		56	85
Avg 83	86	83	83

Phagocytic measurements with rat neutrophiles. Phagocytic activities of rat neutrophiles are presented in Table II. In section A, the procedure employed for tests with human blood was duplicated. Malonate was added to give concentration of 1 mg malonate per ml. Activity of malonate treated blood averages 83%, the control averages 86%. In agreement with tests on human blood, 1 mg malonate fails to alter the activity of blood phagocytes. For section B of Table II, blood was drawn from rats that had previously received 8 injections of malonate. Both experimental and control values average 83%.

Bactericidal activity of blood serum. Table III summarizes experiments designed to determine the bactericidal effect of rat serum. In section A the average bacterial counts of 3 experiments employing normal serum are re-

TABLE III. Number of Viable *Salmonella typhimurium* Present in 3 ml Serum at Times Indicated following Addition of Bacterial Suspension. Section A—normal serum. Section B—serum from rats previously given 2 hourly inj. of 0.5 mg malonate/g body wt. Section C—serum after addition of 3 mg zymosan/ml.

	No. of bacteria/3 ml serum		
	A	B	C
	Avg counts of		
	3 exp.	2 exp.	3 exp.
Hr	Control	Malonate	Zymosan
0	11,111	12,500	12,444
1	2,487	2,200	19,817
2	4,123	3,835	79,333
3	4,267	3,800	255,000
4	5,087	3,900	796,667
5	8,900	5,838	2,076,667

corded. In 1 hour, approximately eight-tenths of the bacteria were destroyed. A slow increase in number is noted between 1 and 4 hours, this increase being more rapid after 4 hours.

The effect of malonate injections on the bactericidal properties of serum is shown in section B. These results are similar to those of control experiments.

Section C gives average bacterial counts from 3 experiments employing addition of zymosan to rat blood serum. An exponential increase in bacterial numbers was obtained throughout the period of observation, indicating absence of any effective antimicrobial property.

Discussion. On the basis of these results, it seems permissible to conclude that malonate, at concentration of 1 mg/ml phagocytic mixture, fails to alter the phagocytic capacity of human or rat blood neutrophiles. At concentrations of 5 and 15 mg malonate/ml phagocytic mixture, an increasingly significant inhibition is observed with human neutrophiles. Taking dosage into consideration, rate of absorption from the peritoneal cavity and rate of excretion of malonate, the concentration of 1 mg malonate/ml phagocytic mixture would seem most closely to approximate conditions present when 8 injections of 0.5 malonate/1 g body weight are administered. With these injections, the capacity of phagocytes to ingest bacteria is not impaired but survival time of rats injected with *S. typhimurium* is significantly reduced. Phagocytic cells must destroy ingested bacteria, if their role in defense is to be fulfilled, but our experiments offer no evidence of the effectiveness of this important process. Only the initial step in cellular defense is evaluated and that appears unimpaired by malonate in agreement with earlier observations on absence of effect of malonate on uptake of thorotrast by liver, spleen, and lungs of mice(7).

Bactericidal tests with rat serum imply that decreased survival time cannot be correlated with impairment of this function. The destruction and reproduction of bacteria in serum from animals treated with malonate is similar to that from control animals. That all the *S. typhimurium* are not killed by the serum and subsequent multiplication ensues, indicates that the strain of bacteria employed is relatively resistant. The only evidence that the observed bactericidal activity of serum is due to properdin is loss of antimicrobial action that accompanies addition of zymosan. According to Pillemer, *et al.*(11), zymosan inactivates the properdin system.

Summary. Our data fail to implicate an alteration in capacity of blood neutrophiles to ingest bacteria or in the bactericidal property of blood serum as a possible explanation for the mechanism by means of which malonate injections reduce survival time of experimentally infected rats.

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Immersion Hypothermia: Effect of Glycine. (22464)

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Factors responsible for and methods of preventing ventricular fibrillation during hypothermia have been vigorously pursued of late. The anti-arrythmic effects of many pharmacologic agents have been investigated(1,2), but little attention has been paid to the use of metabolic stimulants in hypothermia. Such substances may not directly prevent the onset of ventricular fibrillation, but they may prolong the time required to approach a lethal hypothermic level by increasing heat production within the body. Thus the survival chances of individuals accidentally exposed to hypothermic conditions would be greatly enhanced. An effective metabolic stimulant would also be of therapeutic value during the rewarming process by its ability to aid in rapid reattainment of normal body temperature.

Proteins are known to exert a definite thermogenic effect due to high specific dynamic action of certain amino acid constituents, principally phenylalanine, tyrosine, and glycine(3). The practical applicability of this phenomenon was demonstrated by Gubner, DiPalma, and Moore in patients with peripheral vascular diseases(4). These workers observed that oral ingestion of glycine induced a marked rise in oxygen consumption, heat production, and blood flow. It therefore appeared that glycine might be able to alter rate of cooling and rewarming during and following exposure to acute immersion hypothermia. This present study was executed in an effort to demonstrate a possible thermogenic effort of glycine at low body temperatures.

Methods. Twenty experiments were carried out on 10 apparently healthy mongrel dogs of both sexes, ranging from 10-26 kg. All dogs were anesthetized with 30 mg/kg of intravenous pentobarbital and then immersed in iced water bath of 8°C in the manner described by Hegnauer, *et al.*(5). Shivering was inhibited by appropriate administration of 50-100 mg of pentobarbital. Animals were cooled to rectal temperature of 28°C at which point the cold water was drained. They were exposed then to room air until such time as the rectal temperature fell to 26°C. Rewarming was instituted by immersion in warm water bath of 42-44°C. Rectal, bath, and room temperatures in °C were recorded continuously by means of a Leeds-Northrup iron-constantan potentiometer. During cooling and rewarming the following measurements were made. Oxygen consumption was determined at 35-33°C, 30-28°C, 28-26°C, and during rewarming at 30°C and 35°C. Expired air was collected in a Tissot spirometer and oxygen content measured with Beckman oxygen analyzer. Heat production in Cal./kg/hr was then calculated from oxygen consumption data by the method of Weir(6). Carbon dioxide in expired air was determined with a Haldane-Guthrie gas analyzer and blood sugar levels were measured prior to cooling and at a rectal temperature of 26°C. Continuous electrocardiograms (lead 2) were recorded throughout the experimental procedure. Each dog was rendered hypothermic on 2 separate occasions, separated by 2 to 5 days. On first exposure 5 dogs received 500 cc of 5% glycine solution intravenously, the remaining animals received 500 cc of 5% glucose solution. On the second exposure the so-

lutions were reversed. In each instance the infusion was started 15 minutes prior to immersion and continued until rectal temperature of 26°C was achieved. At beginning of the rewarming phase those animals which had received glycine while being rendered hypothermic were switched to glucose, and vice versa. During rewarming 300 cc of each solution was administered. The procedure of transferring solutions at start of the rewarming phase was carried out in an effort to evaluate the thermogenic property of glycine in non-treated subjects who already had been made hypothermic.

Results. Intravenous administration of 5% glycine solution significantly prolonged the cooling time in dogs subjected to immersion hypothermia (Fig. 1). Average time required to lower the rectal temperature of 10 control dogs from 38.3°C to 26°C was 135.2 minutes. These same animals when receiving glycine required 169.8 minutes to cool from 38.2°C to 26°C, an increase of 34.6 minutes ($p = <0.01$). The difference in cooling rates between the two groups is reflected in the significantly greater heat production (Cal./kg/hr) of dogs receiving glycine (Fig. 2). As indicated in Fig. 2 glycine exerted its most marked effect at rectal temperature of 30-28°C ($p = <0.05$). As the animals cooled further, the difference between the 2 groups became negligible. Measurements of blood sugar revealed a significantly higher concentration at 26°C than at 38°C in the glucose treated dogs, as expected, and also in the

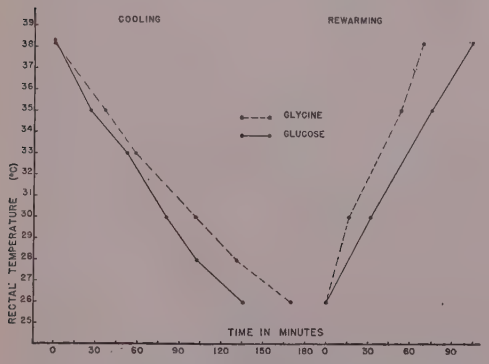

FIG. 1. Total cooling and rewarming time in glucose and glycine treated dogs.

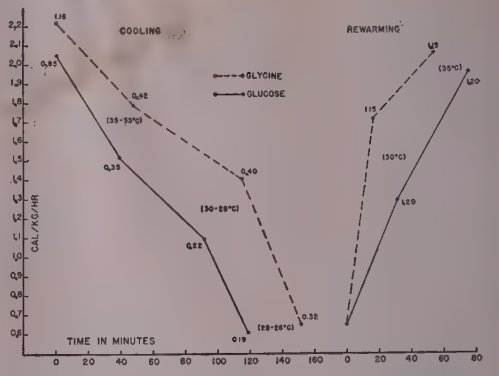

FIG. 2. Heat production in cal./kg/hr in glucose and glycine treated dogs during cooling and rewarming. Figures above and below points indicate stand. dev. Rectal temperatures in °C are presented in the parentheses.

group receiving glycine ($p = <0.01$). The latter is due perhaps to a depletion of liver glycogen as demonstrated by Peterson, *et al.* (7).

During the rewarming phase the marked thermogenic ability of intravenous glycine was illustrated. Those dogs in which the glycine infusion was started at 26°C reattained normal body temperature in average time of 69.7 minutes. These same dogs not treated with glycine during the rewarming phase required 104 minutes to achieve a rectal temperature of 38°C. The difference in rewarming time of 34.3 minutes was significant at the 0.01 level. The heat production calculated from the oxygen consumption again was greater in glycine treated group during rewarming. However, the scatter of values was considerably greater and so the difference between the 2 groups did not attain statistical significance ($p = >0.05<0.1$). The failure of the latter to achieve significance most likely was due to the residual effect of glycine in those dogs which were switched from glycine to glucose at the start of the rewarming phase.

An effort was made to quantitate the increase in heat production due to the administration of glycine and to compare this with the increase anticipated on the basis of the specific dynamic action of glycine alone. Two methods were used to calculate the heat pro-

duction which can be directly attributed to glycine thermogenesis: 1) The difference in heat content between each group after 135 minutes of immersion (average time required for controls to cool to 26°C). The heat content of each group was determined according to the formula: Caloric content = Mass X Specific heat X Mean body temperature(8). As calculated by this method the caloric content of the glycine group was 14.16 calories greater than controls. 2) The difference between the total caloric production of each group during 135 minutes of hypothermic exposure. The total caloric content for each group was determined by measuring the area under the respective curves in Fig. 2. Utilizing this technic the glycine treated dogs showed a total caloric production of 40.9 calories. The glucose group produced an average of 28.4 calories. Thus the caloric difference as determined by this method was 12.5 calories. Therefore, there is good agreement in 2 methods used to determine the caloric difference between groups.

During the 135 minute exposure period approximately 20 g of glycine had been infused. Assuming that glycine has a caloric content of 4 calories/g and a specific dynamic action of 30%, the maximum increase in heat production that can be attributed to the specific dynamic action of glycine would be 24 calories. Therefore, the increased caloric content of the glycine treated dogs can be explained solely on the basis of the specific dynamic action of glycine.

Discussion. The results presented herein indicate that agents which increase thermogenesis may be of practical importance in hypothermia. Glycine appears capable of delaying the rapidity with which a lethal hypothermic state is attained and of augmenting the rate of rewarming in dogs already rendered hypothermic. It is interesting in this regard to note that the relatively small caloric increase of 12-14 calories was sufficient to cause a significant difference in the cooling time of the two groups. Possibly the administration of larger quantities will exert an even greater effect. This is true, of course, only if the heat loss does not increase concomitant with the increase in heat production. Gubner, *et al.*(4) reported that 20 g of glycine per orum induced in human patients a rise in oxygen consumption and also a marked peripheral vasodilatation. The latter indicates a rapid dissipation of the extra heat produced by glycine. Under hypothermic conditions this avenue of heat loss is minimal, and so thermogenic agents should provide an effective adjunctive means of therapy in accidental and induced hypothermia.

The exact mechanism responsible for the extra heat production attributed to glycine is difficult to evaluate. As stated previously the increased caloric production can be adequately explained on the basis of the specific dynamic action of glycine if we assume that the SDA of amino acids is the same at low body temperatures. No evidence is available at present on this particular subject. Amino acids such as glycine are reported to possess a definite hyperglycemic effect. The significant rise in blood sugar observed in the glycine group supports this thesis. Petersen, *et al.*(7) have shown that glycine has a metabolic adrenergic effect, depletes liver glycogen and so produces hyperglycemia. This breakdown of glycogen to glucose which is an exothermic reaction may be responsible in part for the extra heat production observed in the glycine group. It is not certain, however, whether the latter should be considered a part of the specific dynamic action of amino acids or an entirely separate phenomenon.

Summary. The intravenous administration of a 5% glycine solution caused a significant increase of 34.6 minutes in the time required to lower the rectal temperature of dogs from 38°C to 26°C. Total rewarming time was decreased by 34.3 minutes in the glycine treated group. The differences in cooling and rewarming rates between the treated and nontreated animals was due to the increased heat production observed in the dogs receiving glycine. The possible applicability of thermogenic agents in accidental hypothermia is discussed.

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Action of Butyltolylsulfonylurea on Liver Glycogenolysis.* (22465)

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The hypoglycemic action of a sulfonamide derivative, sulfonamido-isopropylthiadiazole, was first discovered by Janbon and coworkers in 1941(1). Loubatieres(2) suggested that this compound caused hypoglycemia by stimulating insulin secretion. A similar conclusion was reached by Chen and coworkers(3) who investigated the hypoglycemic action of sulfanilamido-cyclopropyl-thiazole. It is possible that one mechanism by which the sulfonamide produces hypoglycemia is by inhibiting the rate of degradation of insulin, as has been reported by Mirsky(4) and Williams(5) to occur with certain other sulfonamides. Holt and coworkers(6) reported that sulfonamido-cyclopropylthiadiazole produces morphologic changes in the alpha cells of the pancreas in rabbits and they suggested that the compound caused hypoglycemia by inhibiting glucagon secretion. Franke and Fuchs(7), Bendfeldt and Otto(8), and Achelis and Hardebeck(9) attributed the hypoglycemic action of butylaminobenzenesulfonylurea to decreased production of glucagon. However, Tyberghein and Williams(10) found that extracts of serum of rabbits treated with butyltolylsulfonylurea (BTSU), which also produces hypoglycemia, had the same glycogenolytic stimulating activity in liver slices as extracts of serum of normal rabbits. Since there was good indication that this glycogenolytic effect was due to glucagon, these observations weaken the hypothesis of a decreased glucagon secretion. Therefore another mechanism was sought to explain the hypoglycemic effect of the sulfonamide preparations. An action on liver glycogen has been discussed by Miller and Dulin(11) and by Loubatieres(12). In the present study we investigated whether or not the hypoglycemic action of BTSU† could be related to an inhibition of glucose release by the liver and whether enzymes involved in glycogenolysis were affected by BTSU.

Methods. Some studies were conducted with rats and others with rabbits.

Rat Experiments. The effect of BTSU on blood sugar and liver glycogen was studied in Sprague-Dawley rats, each weighing approximately 300 g, prepared in one of the 2 following ways: (a) fasted for 24 hours, or (b) fed a standard amount of glucose for 24 hours. One group was fasted since it was believed that in this manner an inhibitory effect of BTSU on glycogenolysis might be more apparent, since normally the glycogen stores are considerably depleted after 24 hours. In all experiments with rats 80 mg of the sodium salt of BTSU, dissolved in 2 ml of water, was given every 12 hours by stomach tube. The first dose coincided with the beginning of the

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TABLE I. Effect of BTSU on Blood Glucose and Liver Glycogen in Rats.

	No. of animals		Blood glucose, mg %		Liver glycogen, mg/g tissue	
	Controls	Treated BTSU	Controls	Treated BTSU	Controls	Treated BTSU
Fasted 24 hr	15	15	84 ± 8.4*	49 ± 4.3*	1 ± 1.6*	16.3 ± 4.3*
Fed glucose	8	8	106 ± 8.2	68 ± 5.6	50.5± 6	53.8 ± 3.9

* Stand. error of the mean.

fasting period or of the glucose feeding. The animals were sacrificed between 2 and 3 hours after the third dose. Blood was drawn for glucose determination(13) and the liver excised for glycogen determination(14). In the group of rats fed only glucose, 500 mg was given every 12 hours by stomach tube with or without BTSU.

The effect of BTSU on glucose-6-phosphatase activity was studied in rat liver homogenates. Liver homogenates were prepared from normal fasted rats and from fasted rats given three doses of BTSU, 80 mg each, by stomach tube, according to the method described earlier. Aliquots of the homogenates were incubated at 28.9°C with .08 M glucose-6-phosphate in 0.1 M citrate buffer, pH 6.45 (15). The formation of inorganic phosphate was measured at the end of 30 minutes incubation(16).

Rabbit Experiments. Studies were conducted in rabbits on the effect of BTSU given orally on the glycogenolysis of surviving liver slices. New Zealand white male rabbits, weighing approximately 2 kg were given 500 mg of BTSU every 12 hours for 4 days except for the first and the last dose which were 1 g each. The animals were sacrificed about 3 hours after the last dose was given. Liver slices, weighing approximately 100 mg each, were cut promptly and put in 20 ml beakers containing 2 ml of buffer solution (1 volume 0.1 *N* potassium phosphate buffer and 4 volumes 0.9% sodium chloride solution), pH 7.5; the mixture was incubated at 37° for 45 minutes.

Results. The data presented in Table I indicate that in fasted rats given BTSU the amount of liver glycogen is much greater than in fasted control rats. In rats fed only glucose for 24 hours the amount of glycogen in the liver was the same whether or not BTSU was given. In both the fasted and the glucose-fed rats there was considerable hypoglycemia in the BTSU-treated groups. The conversion of glycogen to glucose is probably impaired since there presumably was relatively little synthesis of glycogen in these animals. The finding that the glycogen content of the glucose-fed rats is the same with or without BTSU treatment, indicates that the conversion of glucose to glycogen is not significantly impaired by BTSU feeding. Data indicating that BTSU decreases liver glycogenolysis *in vitro*, also, are presented in Table II. The glucose output from liver slices of rabbits treated with BTSU was less than that from liver slices of control rabbits. It is of interest to note that this effect was obtained only if the BTSU was given to the animals before sacrifice. The addition of BTSU to the incubation medium *in vitro* did not significantly decrease the glucose output from liver slices of normal rabbits.

TABLE II. Effect of BTSU Treatment in Rabbits on Spontaneous Glucose Output from Liver Slices.*

Spontaneous glucose output, mg/100 mg liver tissue		% decrease in spontaneous glucose output by BTSU treatment
Controls	Treated BTSU	
1.17†	.97†	18
1.54	1.14	26
1.17	.94	19
1.33	1.02	23
1.39	1.18	16
1.51	1.18	22
1.45	1.08	21

* 2 rabbits used in each experiment.
† Mean value for 3 different slice preparations.

If BTSU blocked any step in glycogen synthesis one would expect a decrease in glycogen content in BTSU-treated, glucose-fed rats. This was not found. That there was not an increase in the liver glycogen in these rats as compared to those fed glucose only (despite

TABLE III. Effect of BTSU Treatment on Glucose-6-phosphatase Activity of Rat Liver Homogenates.

	μM inorganic phosphate liberated from glucose-6-phosphate in 30 min./mg protein*	
	Exp. I	Exp. II
Controls	.46 ± .03 (4)†	.48 ± .02 (3)
BTSU treated	.38 ± .01 (4)	.38 ± .02 (4)

* Determined by biuret method; protein values for treated animals averaged ca 95% of those for controls.

† Refers to No. of animals.

the hypoglycemia existing) may be explained by the fact that the amount of glucose given was sufficiently large to the make breakdown of liver glycogen of minor importance as compared to synthesis of liver glycogen. In this connection, it may be noted that the amount of glycogen in the glucose-fed rats was larger than in rats fed *ad lib.* (50.5 ± 6 compared to 35.3 ± 3 mg per g). The results described above are consistent with the hypothesis that BTSU-hypoglycemia is the result of a reduced rate of liberation of free glucose from liver glycogen. Since no obvious impairment of glycogen deposition was found, it seems reasonable to suppose that the irreversible step in glucose liberation is involved, *i.e.*, the reaction glucose-6-phosphate → glucose + phosphate.

To determine whether the glucose-6-phosphatase activity of BTSU-treated rats was reduced, compared to controls, the activity of liver homogenates in liberating inorganic phosphate from glucose-6-phosphate was measured. Table III contains data indicating a significant depression of the activity of this enzyme. Similar observations have been made by others(17). One mode of action of BTSU appears, therefore, to be a decrease in the activity of glucose-6-phosphatase, resulting in a decreased release of glucose by the liver. It is possible that this effect of BTSU is the result of a stimulation of insulin secretion or of decreased insulin degradation, caused by the drug. However, this effect of insulin requires 6-12 hours after insulin administration is begun(18), while preliminary experiments indicate that the effect of BTSU on glucose-6-phosphatase activity is more rapidly established.

Summary. 1. In fasted rats butyltolylsulfonylurea (BTSU) produces hypoglycemia and decreased liver glycogenolysis. 2. In rats fed glucose only, BTSU also produces hypoglycemia but the amount of liver glycogen is comparable to that of untreated glucose-fed rats. 3. After treatment of rabbits with BTSU for four days, the spontaneous glucose output from liver slices was less than from liver slices of control animals. 4. The formation of inorganic phosphate by liver homogenate in the presence of glucose-6-phosphate is less in the BTSU-treated rats than in control rats, indicating that the glucose-6-phosphatase activity is less in the BTSU-treated rats than in control rats. 6. These observations tend to indicate that BTSU produces hypoglycemia at least partially by decreasing glucose-6-phosphatase activity in the liver.

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Study of Different "Fibrinoids" by Histochemical Means.* (22466)

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The so-called fibrinoid degeneration occurs in a variety of pathological conditions. It is characterized by the appearance of a homogeneous, strongly acidophilic material which is stained by Weigert's fibrin and the P.A.S. methods. The problem whether fibrinoid change is due to impregnation of the tissue by fibrin, or represents a change in the connective tissue ground substance has not yet been settled definitely(1-4); neither is it clearly known whether the "fibrinoid materials" differ in the different pathological states in which they occur (with some common characteristics which are responsible for the common staining reactions), or whether it is a single compound appearing in a variety of diseases(5,6).

The present report deals with attempts to answer these two questions by a study of the histochemical characteristics of fibrinoid materials present in different diseases.

Materials and methods. Formalin fixed and paraffin embedded human autopsy and biopsy material was used. The choice of material was casual and bore no relationship to etiological and pathogenetic factors. Normal fibrin was included for comparison with the "fibrinoids." The following specimens were included in all experiments. (1) Mural thrombus of heart; (2) three month placenta; (3) placenta at term; (4) nephrosclerosis with signs of malignant hypertension; (5) heart in systemic lupus erythematosus; (6) kidney in periarteritis nodosa; (7) pinguecola in the conjunctiva. Other examples of fibrinoid change were included originally in the series, but owing to the small size of blocks they had to be subsequently discontinued. Serial sections 8 μ in thickness, were made from all the blocks. The different staining and histochemical blocking procedures were performed simultaneously on slides of each tissue. The following staining methods were used: a) H. & E.; b) Weigert's fibrin method; c) Mallory's Azan; d) periodic acid Schiff method (P.A.S.); e) Rinehart and Abul Haj's modification of Hale's colloidal iron method; f) ninhydrin-Schiff method; g) performic-acid-Schiff; h) Sudan black; i) Weil-Weigert's myelin method; j) mucicarmin. The effect of the following histochemical blocking reactions and treatments on staining by the 4 first mentioned staining procedures was studied; a) acetylation for 24 hours at 37° with an acetic anhydride-pyridine mixture; b) deacetylation of the acetylated sections; c) deamination by van Slyke's nitrous acid reagent; d) deamination by ninhydrin; e) extraction of sections for 24 hours by 1% acetic acid; f) extraction for 24 hours by pyridine; g) extraction for 24 hours by 1% sodium carbonate. All the histological and histochemical procedures were performed as suggested by Lillie(7).

Results. Table I summarizes the differences in staining of the various "fibrinoids" by the ninhydrin-Schiff and the colloidal-iron staining reactions.

The ninhydrin-Schiff staining reaction indicates the relative concentration of protein matter (as represented by α amino acids) within the "fibrinoids," while the colloidal iron reaction visualizes acidic groups which probably belong to mucopolysaccharides. With the fibrin in the mural thrombus serving as control, it can be deduced that the eosinophilic material surrounding the villi and the Nitabuch layer in both the young and mature placentas contains protein in a similar concentration to that of fibrin. The "fibrinoid" of renal arteriosclerosis and of periarteritis nodosa appears to contain more protein material, while that of pinguecola contains much less protein than the fibrin. With the colloidal iron technic more marked differences can be noted between the fibrin and all the

* This study was supported by grant from Hadassah Medical Organization.

TABLE I. Staining of Different "Fibrinoids" by the Ninhydrin-Schiff and the Colloidal Iron Technics.

			Lupus erythematosus			Mature placenta		Incomplete abortion		
	Pinguecola	Periarteritis nodosa	"Fib'n" on pericardium	"Fibrinoid" in artery	Renal arteriolosclerosis	"Fibrin" around villi	Nitabuch's layer	"Fibrin" around villi	Nitabuch's layer	Fibrin in mural thrombus, heart
Ninhydrin-Schiff	±	4+	?	?	4+	2+	2+	2+	2+	2+
Colloidal iron staining	4+	2+	?	?	2+	3+	3+	3+	3+	+

Note: Intensity of the staining of "fibrinoids" was graded as: 0 = no staining; ± = staining barely visible; + = weak; 2+ = moderate; 3+ = marked; 4+ = intense staining.

"fibrinoids." The pinguecola "fibrinoid" appears to contain most acidic groups, while that of renal arteriosclerosis and of periarteritis nodosa does not differ much from fibrin. The difference in staining by colloidal iron between the Nitabuch layer and the "fibrin" which surrounds the placental villi on the one hand, and the fibrin of the thrombus on the other, is remarkable.

It has been stated that acidophilia is due to the presence of free amino groups(8). Accordingly, a study was made of the effect of reagents which block or destroy the amino groups, on the staining of different "fibrinoids" with the H.&E. and the Azan staining methods. It can be seen in Table II that the effects of the blocking agents were comparable, although not identical, for both staining methods. Treatment by van Slyke's reagent was the most effective in blocking acidophilia. It inhibited completely the staining of fibrin and of the "fibrin" around the villi, the "fibrin" of the pericarditis in Lupus erythematosus, and of the "fibrinoid" of pinguecola. The treatment did not completely block, however, the acidophilia of the other "fibrinoids." Treatment by ninhydrin was less effective in blocking acidophilia, but the materials which behaved like fibrin were the same for both blocking methods. Acetylation was less effective than van Slyke's reagent, but more effective than ninhydrin in abolishing acidophilia. The four compounds, which behaved like fibrin towards the other 2 amine-blocking procedures after acetylation, stained with the same intensity as fibrin.

Two materials were affected differently from the others by the amine blocking procedures. (1) The "fibrinoid" of pinguecola, which behaved like fibrin towards the van Slyke reagent, was not affected by the acetylation. (2) The acidophilia of the "fibrinoid" of periarteritis nodosa was affected to the same extent by ninhydrin treatment as by

TABLE II. Effect of Varied Treatments as Indicated by Subsequent Staining of Different "Fibrinoids" by Acid Dyes (H. & E. and Azan's Methods).

			Lupus erythematosus			Mature placenta		Incomplete abortion		
Effect of	Pinguecola	Periarteritis nodosa	"Fib'n" on pericardium	"Fibrinoid" in artery	Renal arteriolosclerosis	"Fibrin" around villi	Nitabuch's layer	"Fibrin" around villi	Nitabuch's layer	Fibrin in mural thrombus, heart
A*	4+	2+	2+	4+	3+	2+	3+	2+	3+	2+
A and D*	4+	4+	2+	4+	3+	2+	3+	2+	3+	2+
Van Slyke's R*	0	2+	0	2+	+	0	2+	0	2+	0
Ninhydrin R*	3+†	2+†	3+†	3+†	4+†	3+†	4+†	3+†	4+†	3+†

Note: Intensity of the staining of "fibrinoids" was graded as: 0 = no staining; ± = staining barely visible; + = weak; 2+ = moderate; 3+ = marked; 4+ = intense staining.

* A = Acetylation; D = Deacetylation; R = Reagent.

† Findings with Azan; effect on eosin staining was much more pronounced.

TABLE III. Effect of Varied Treatments as Indicated by Subsequent Staining of Different "Fibrinoids" by Weigert's Fibrin Method and by the P.A.S. Procedure.

Effect of	Pinguecola	Periarteritis nodosa	Lupus erythematosus: "Fib'n" on pericardium	Lupus erythematosus: "Fibrinoid" in artery	Renal arteriolosclerosis	Mature placenta: "Fibrin" around villi	Mature placenta: Nitabuch's layer	Incomplete abortion: "Fibrin" around villi	Incomplete abortion: Nitabuch's layer	Fibrin in mural thrombus, heart
A*	0	0	0	0	0	0	0	0	0	0
A and D*	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Van Slyke's R* on fibrin stain	4+	4+	4+	4+	3+	3+	3+	4+	4+	3+
Van Slyke's R* on P.A.S.	+	2+	4+	4+	4+	3+	3+	3+	3+	2+
N* on fibrin stain	3+	2+	?	?	2+	0	2+	0	+	0
N* on P.A.S.	+	2+	+	3+	4+	2+	4+	+	3+	+

Note: Intensity of the staining of "fibrinoids" was graded as: 0 = no staining; ± = staining barely visible; + = weak; 2+ = moderate; 3+ = marked; 4+ = intense staining.
* A = Acetylation; D = Deacetylation; R = Reagent; N = Ninhydrin.

van Slyke's reagent. It was also affected by acetylation to the same extent as fibrin.

The "deacetylation" of acetylated sections had no effect on acidophilia in most materials. The only marked effect in restoring acidophilia was noted in the "fibrinoid" of periarteritis nodosa. It should be noted, though that no such effect could be studied where the acetylation itself had no effect on acidophilia.

Table III shows the results of different procedures on the P.A.S. and Weigert's fibrin staining methods. It is noteworthy that Weigert's method reacted similarly to P.A.S. towards the blocking agents. As both methods were completely blocked by the acetylation procedure and could be reversed by deacetylation, it is believed that staining by Weigert's fibrin method might be due to the presence of 1.2 glycols or 1-glycol-2-amino groups. The implications of these findings in the problem of Gram staining of bacteria are obvious as the Gram procedure is almost identical with Weigert's fibrin method.

Deamination by van Slyke's reagent did not markedly affect the staining of the "fibrinoids" by Weigert's fibrin stain, but it did influence to different degrees the staining of the various "fibrinoids" by P.A.S. The "fibrinoid" of periarteritis nodosa behaved in this case like fibrin. The "fibrinoid" of pinguecola was even more affected by van Slyke's reagent than fibrin. Van Slyke's reagent had no or little effect in this staining with any of the other "fibrinoids."

By the effect of ninhydrin treatment on both Weigert's fibrin and P.A.S. methods the "fibrinoids" could be divided into 3 groups. The "fibrin" around the placental villi and the fibrin in the pericardium in Lupus erythematosus behaved like fibrin. All the other "fibrinoids" with the exception of that of pinguecola, behaved differently. Again, the "fibrinoid" of pinguecola was unique in its behavior.

No relevant additional findings were obtained by the use of the other staining and blocking methods.

Discussion. The data reported above indicate that the term "fibrinoid" is merely descriptive and covers a number of different compounds. The various "fibrinoids" differed from fibrin to a varying extent. The "fibrin" layers around the placental villi and in the pericardium of Lupus erythematosus were most similar to the fibrin of the mural thrombus. It is possible that we are dealing in this case with fibrin in which a change has occurred. The hypothesis which derives "fibrinoid" from plasma fibrin(3) does not seem to hold for the other "fibrinoids" studied in this work. The common staining characteristics of the different fibrinoids appear to

be due to the presence of saccharidic complexes. The common denominator of all these compounds is their acidophilia and their staining by Weigert's fibrin and P.A.S. methods. These reactions might be due to free amino-groups, many of which are probably in a 2 position in respect to hydroxyls (as the deamination procedures had some effect on the P.A.S. staining). It seems probable, although it has not been proved, that the complexes responsible for the staining reactions of the fibrinoids and fibrin might be different polysaccharides, possibly poly-amino-sugars (1). Our findings corroborate those of other workers who found that "fibrinoids" differed from fibrin in their histochemical(1,6,8,9), physical(10), and biochemical(11) characteristics.

The multiplicity of "fibrinoids" confirms and extends the views expressed by Klemperer(5) and by Gueft and Laufer(12) who maintained that the systemic Lupus erythematosus fibrinoid derives from DNA and is therefore chemically different from the other "fibrinoids."

Summary and conclusions. 1. The types of "fibrinoids" in different pathological processes were studied by means of specific staining reactions and histochemical procedures intended to block amino groups. 2. All fibrinoids differed from fibrin in their behavior. The "fibrin" layer around the placental villi behaved more like fibrin than any other "fibrinoids." The other "fibrinoids" differed markedly from each other and from fibrin. It may be concluded that in spite of their tinctorial similarities, the various "fibrinoids" contain different compounds and might represent the end-stages of different processes.

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Effect of High Hydrostatic Pressures on Colchicine-Damaged Mast Cells of Peritoneal Fluid.* (22467)

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Morphologic changes in the mast cells of rat peritoneal fluid have been induced by subcutaneous injections of colchicine(1). The cells, normally spherical, are altered into irregular masses, sometimes with branched pseudopod-like extensions of cytoplasm, and the nucleus appears to be pushed to the periphery of the granular cytoplasmic mass as if being ejected from the cell. These changes

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are apparent within 3 hours following injection of the drug. Since cells normally round up when suspended in a fluid medium, this unusual behavior of free-floating mast elements in the peritoneal fluid of colchicine-treated rats suggests possible alterations of sol-gel equilibrium within the cytoplasm. The application of high hydrostatic pressure has proved an excellent tool for the study of sol-gel equilibria in ameboid movement(2) and in dividing marine eggs(3). In the present study, high hydrostatic pressure technics have been employed to investigate whether the abnormal, non-spherical shape of mast cells in colchicine-treated rats is associated with a strongly gelated cytoplasm.

Materials and methods. Intact young adult female rats (150 ± 10 g) of a modified Long-Evans strain were given a single subcutaneous injection of freshly prepared colchicine alkaloid (Eimer and Amend, U.S.P. X11) solution in 0.9% NaCl (0.1 mg/100 g body weight). The animals were killed by exsanguination under light ether anesthesia 3-5 hours after the injection. Peritoneal fluid was obtained as described previously(4-6) and 1 volume aliquots were added to approximately 40 volumes of freshly obtained rat serum diluted 1:1 and 1:2 with 0.9% saline. The apparatus used for subjecting the cells to high hydrostatic pressures has been described(7). The experiments were carried out at 38 ± 1°C. The apparatus is designed to allow uninterrupted direct observation of the cells within the pressure chamber at a magnification of 480 diameters throughout the application of pressure. Specific cells within the microscope field were kept under constant direct observation for the duration of the experimental run. The cells were placed in the pressure chamber immediately upon removal from the peritoneal cavity of the animal and a new rat was used for each determination. A control aliquot of each sample was kept at atmospheric pressure in a hemacytometer for the duration of the experiment and specific mast cells were observed similarly.

Results. Hydrostatic pressures of 8,000 lbs/in^2 maintained for 20 minutes or 10,000 lbs/in^2 maintained for 5 minutes did not affect the morphology of the abnormal mast cells. However, with pressures of 12,000 lbs/in^2, some aberrant mast elements evidenced rounding up within 45 seconds of pressure application. Not all the abnormally shaped cells were affected. In general, forms departing moderately from the normally spherical or ovoid shape were responsive to the application of pressure while the more strikingly polymorphic mast elements appeared but slightly affected by the applied pressure. Some free-hand sketches of the changes observed are reproduced in Fig. 1. In some cases, cells which could not be induced to round up completely under pressure did so when the pressure was released instantaneously from 12,000 lbs/in^2 to atmospheric level. Under these circumstances, cells which had already assumed a spherical shape appeared to shrink slightly. All the control mast cells maintained at atmospheric pressure remained morphologically unaltered throughout the observation periods.

Discussion. Cells freely suspended in a liquid medium generally tend to assume a spherical shape. This rounding is usually attributed to tensional forces at the cell surface, which reach a minimum when the cell is spherical. Moreover, it presupposes that any gel structure in the cytoplasm is weak or absent and that there is no rigid pellicle.

In the peritoneal fluid of young rats, mast cells and other cellular types are normally spherical, whereas in the connective tissue spaces they are polymorphic. This suggests that the tensional forces and gelational states may be altered by contact with solid surfaces. Thus the cells become deformed in accordance with the principles described by Garber (8) and by Weiss and Garber(9) for fibroblasts in tissue culture.

The fact that the mast cells in the peritoneal fluid of colchicine treated animals display a high degree of polymorphism(1) suggests that the drug may have a definite effect upon the plasmagel structure of this cell. Apparently, it initiates the formation of strongly gelled localized cytoplasmic regions which can contract, forcing an outflow of plasmasol into

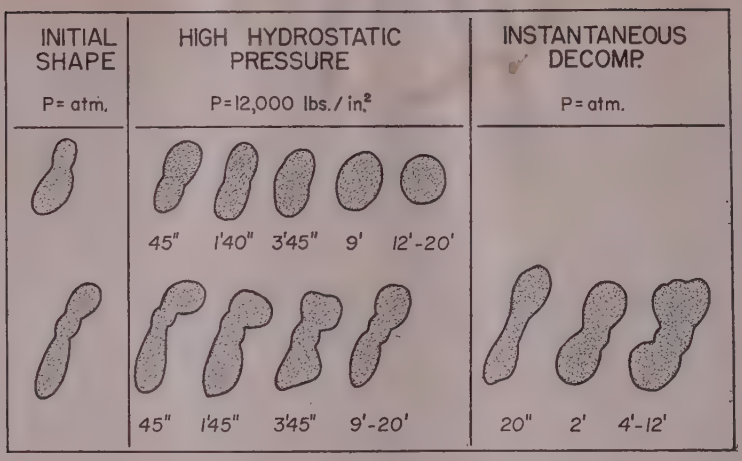

FIG. 1. Free hand sketches of mast cells visualized within the pressure chamber. Left: Optical cross section of 2 cells prior to application of pressure. Center: Consecutive sketches of these 2 cells while under pressure. Right: Changes observed after release of pressure. The upper cell is typical of "responsive" ones while the lower one represents the "unaffected" type. This difference arises presumably from different degrees of initial cytoplasmic gelation.

pseudopodium-like processes, in a manner analogous to ordinary amoeboid movement. This viewpoint, in fact, is well supported by the results of the present experiments. One well established effect of high pressure is to shift the sol-gel equilibrium, causing a drastic solation of the plasmagel system which would permit the cells to reassume their normal rounded form. It seems possible that the more highly aberrant forms are more firmly gelled since these do not become completely rounded even by pressures of 12,000 lbs/in^2. It is probable that even the most abnormally shaped mast cells would round up more completely if higher pressures were applied.

Sol-gel and pressure-volume relations of cells have been discussed in more detail in previous publications(2,10). The effect of colchicine on cytoplasmic viscosity has been investigated by Beams & Evans(11) for *Arbacia* eggs. They report a definite lowering of cytoplasmic viscosity at the interior of the cell with a probable, although less evident, effect at the cell surface. Colchicine does not inhibit locomotion in *Amoeba* or cyclosis in *Tradescantia*, both of which presumably depend on sol-gel relations(11). Wilbur(12) has suggested that colchicine affects cytoplasmic viscosity of *Arbacia* eggs indirectly "by inhibiting processes which do produce viscosity changes."

Mast cells are not generally considered to undergo mitosis. Therefore, the reasons for their sensitivity to colchicine both *in vivo*(1) and *in vitro*(13) as well as to podophyllotoxin (another mitotic inhibitor) under similar conditions(13) remain to be elucidated.

Summary and conclusions. Abnormally shaped mast cells of peritoneal fluid in colchicine-treated rats are affected by the application of high hydrostatic pressure. The changes observed under pressure include rounding up of the cell, suggesting that the abnormal morphology is maintained by a strongly gelated cytoplasm. Induction of localized foci of gelation by colchicine is postulated to explain the formation of polymorphic mast elements.

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Induced Accumulation of Citrate in Therapy of Experimental Lead Poisoning.* (22468)

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(Introduced by A. M. Brues.)
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Chelating or complexing agents are widely used to counteract the toxicity of metal ions and to accelerate the elimination of radioelements from the body. Since the distribution of administered water-soluble chelating agents is primarily extracellular it is unlikely that these chelating agents are in sustained contact with cellular components. For this reason we have explored the possibility of interfering with metabolic processes *in vivo* so that certain compounds which are naturally present in the body and which possess chelating or complexing properties might be maintained in abnormally high concentrations within the cells and thus be utilized to modify metal toxicity. Citric acid is a compound which is intimately involved in the Krebs cycle and which possesses complexing properties. Citrate ion, Cit^{3-}, forms a stable complex with Pb^{++} ($PbCit^{-}$) under physiological conditions; the logarithm of the formation constant for this reaction is 5.74(1). For these reasons, lead was chosen as the toxic agent and citrate as the metabolic chelating agent. Sodium citrate in clinical use has resulted in symptomatic relief in some cases of lead poisoning(2-4). However, it was necessary to administer large doses at frequent intervals over fairly long periods of time, because citrate ion, administered either orally or parenterally, disappears rapidly by excretory and metabolic pathways(5). These facts suggested that an induced maintenance of high citrate levels *in vivo* might be of greater therapeutic value than the repeated administration of exogenous citrate(6). Administration of sodium fluoroacetate (FA) in lethal doses has been shown by Peters *et al.*(7) to produce synthesis of fluorocitric acid which acts as a competitive inhibitor in the enzymatic destruction of citric acid in the tissues(8-10). It has been shown(6) that a single injection into rats of a small, non-lethal dose of FA causes a pronounced accumulation of citric acid in many tissues though not in blood. While the control levels of citrate in the kidney and spleen were roughly 20 μg and 70 μg per gram fresh tissue respectively, maximum levels of more than 10 times normal were reached in the kidney, spleen, and heart within 4 to 6 hours and high levels were maintained for several more hours.

In the present experiment the survival of animals given toxic doses of Pb^{++} ion and treated with small, sublethal amounts of fluoroacetate has been compared with that of citrate-treated and untreated animals.

Materials and methods. The animals used were 78- to 181-day-old Sprague-Dawley female rats weighing between 225 and 270 g. In each series of experiments animals were

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TABLE I. Effect of Citrate on Survival of Pb^{++} Poisoned Animals, 4 Groups.

Treatment post Pb^{++} 1 min.	3 hr	6 hr	Survival	Time to 50% death (days)
C*	0	0	0/10	.63
C	C*	0	0/10	.67
C	C	C*	1/10	.67
S*	S*	S*	2/10	.79

* C = Citrate at 250 mg/kg; S = Saline.

evenly selected from 2 age groups; differences between these 2 groups were not statistically significant in terms of the experimental results. The animals were maintained on a diet of Purina chow and water *ad libitum* throughout the experiment. They were grouped together in such a manner as to minimize "cage effects." Lead was administered to all animals in a single injection into the tail vein as a 6.4% solution of lead nitrate ($Pb(NO_3)_2$), in doses ranging from 50-70 mg/kg as Pb. The FA-treated animals received intraperitoneal injections of a 0.005% solution of sodium monofluoroacetate (0.30 mg/kg per injection) at 22 and 5 hr prior to and at 8, 19, and 25 hr after the lead injection (a total of 1.5 mg/kg as FA over a 3-day period). Preliminary experiments indicated that this dosage schedule provided the maximum degree of protection with the minimum of toxic effects. The citrate-treated animals received intraperitoneal injections of 3% sodium citrate solution (250 mg/kg) after the lead injection at the time intervals listed in Table I. Control animals received saline intraperitoneally in equivalent volumes at corresponding time intervals.

Results. Three series of experiments were performed. In the first, 30-day survival was compared in FA-treated and untreated animals following an approximate LD_{90} dose of lead nitrate (70 mg/kg as Pb). The 2 groups (40 treated animals, 70 untreated) each contained an equal number of animals aged 114 and 160 days. The survival in the FA-treated group was 53%, while that in the untreated (saline control) group was 10%. The difference in survival data for the 2 groups was statistically significant: $P << 0.01$. There was no significant difference in time to 50% death, which was 1½ days.

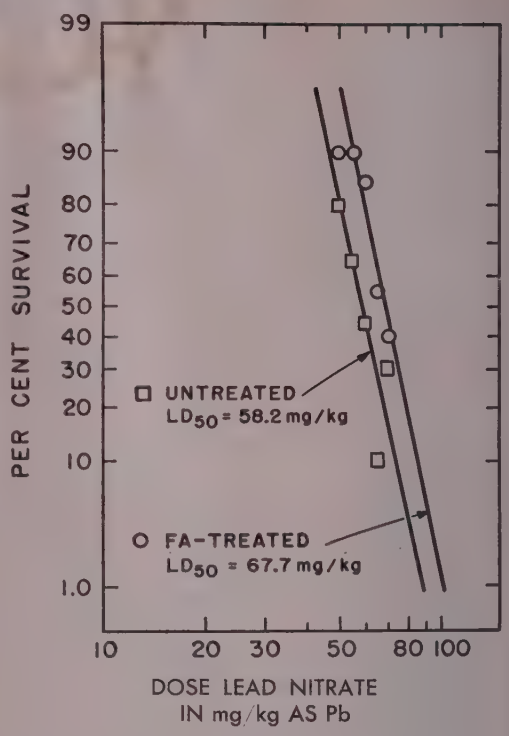

FIG. 1. Survival curves of fluoroacetate-treated and untreated (saline control) groups. Each point represents data from 20 animals except at the lowest dose for the control group and the 2 lowest for the FA-treated, where 10 animals were used.

The second series of experiments was designed to evaluate the shift in the LD_{50} value of Pb for the FA-treated as compared with the untreated. One hundred and seventy 78- and 136-day-old rats were used. Probit curves of the survival data and the LD_{50} values for the FA-treated and untreated groups (Fig. 1) reveal an increase in the LD_{50} of lead nitrate from 58.2 mg/kg (± 4.3%) for the untreated (saline controls) to 67.7 mg/kg (± 3.1%) as Pb for the FA-treated. This difference is significant at the 5% level ($.05 > P > .02$). The curves show no significant difference in slope ($t_b = 0.32$).

A third experiment was performed to determine the effect of repeated intraperitoneal administration of citrate. Animals (181-day-old) were all given lead nitrate (60 mg/kg as Pb) followed at various time intervals by citrate. The results (Table I) reveal no sig-

nificant differences in survival among these groups, and for this reason no further survival studies were undertaken with administered citrate.

Discussion. The similarity in slope of survival curves obtained from the FA-treated and untreated animals supports the explanation that the mechanism of protection is a simple one in which the concentration of toxic lead ions is lowered by the *in vivo* formation of the complex $PbCit^-$. Even though only partial protection was obtained by treatment with FA, the experimental results demonstrate the feasibility of utilizing an induced *in vivo* stimulation of a naturally occurring chelating or complexing agent for the treatment of metal poisoning.

It is conceivable that other metabolic inhibitors may be even more effective in poisoning by lead and other metal ions, since they may induce the accumulation of other naturally occurring chelating agents with more favorable or more prolonged tissue distributions relative to that of the metal. In the present experiment, gross observations at autopsy and hematuria indicated that kidney damage contributed to the death of the lead-poisoned animals. Thus it is possible that the major protective effect of FA resulted from the elevation of intracellular citric acid levels in the kidney. It can be estimated from earlier data (6) that the citrate concentrations in the FA-treated animals were maintained at about 0.5 mg/g of spleen and about 0.3 mg/g of kidney through the course of treatment, but remained near normal levels (~0.03 mg/g) in the blood. Thirty minutes after the injection of 250 mg/kg of sodium citrate the blood citrate level was about 0.6 mg/g whole blood but returned to the normal level within 1½ hours; all other tissue levels remained essentially unchanged. Sustained high citrate levels in the blood have been reported following injection of guanidine compounds in rabbits(12), but we were unable to reproduce this result in rats. Metabolic inhibitors which would induce the accumulation of naturally occurring chelating agents with stronger formation constants for the toxic metal under physiological conditions(11) would also be more effective.

Summary. The concept of interference in a metabolic cycle as a means of modifying metal toxicity has been tested. The accumulation of citric acid in certain soft tissues of the rat has been induced by administration of small, non-lethal doses of sodium fluoroacetate. This has been found to give partial protection to rats acutely poisoned with lead nitrate. Of rats given the LD_{90} of lead nitrate, 53% survived when treated with sodium fluoroacetate. The LD_{50} of lead nitrate was increased from 58.2 mg/kg (as Pb) in saline controls to 67.7 mg/kg in fluoroacetate-treated rats.

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Specific Organ Defects in Early Chick Embryos Following Inoculation with Influenza A Virus.* (22469)

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Characteristic defects in the development of early chick embryos following inoculation of influenza A virus have been reported by Hamburger and Habel(1) and by Shear *et al.* (2). The defects consisted chiefly of micrencephaly, abnormal axis twists and impairment of growth of the amnion. However, Hamburger(3) and Kung(4) observed, in microscopic studies of the abnormal embryos, that development of the brain tissue itself appeared normal and that the flattening of the encephalon, resembling a micrencephaly on gross examination appeared to result from collapse of the ventricles of the brain. The authors attributed the collapse of the ventricles to a lack of ventricular fluid, lost possibly through areas of local injury. Specific organ defects, such as those described following Newcastle disease virus(5-8), were not reported as characteristic effects of influenza A infection. It is the purpose of this paper to describe conditions under which influenza A produces characteristic gross defects of the lens and auditory vesicles in early chick embryos, and in a few instances, neural tube defects.

Material and methods. Virus preparation. The PR8 strain of influenza A virus, grown from dilute inocula in the allantoic cavity of 12-day chick embryos for 48 hours, served as stock virus suspension. The infectivity titer was determined by inoculating 10-fold dilutions of virus suspension intra-allantoically into 12-day embryos and testing after 4 days for virus growth as indicated by agglutination of chicken erythrocytes by the allantoic fluids. The EID_{50} was calculated by the method of Kärber(9). For inoculation into the early chick embryos the suspensions were diluted in buffered saline (pH 7.2) to the required infectivity titer. *Inoculation of embryos.* Eggs incubated in a forced draft incubator at 98-99°F for 45 to 60 hours were prepared with openings and inoculated under the vitelline membrane, over the blastoderm(6) with 0.05 ml amounts of virus suspension. *Determination of stage of development.* Developmental stages for embryos of each group inoculated were determined, according to the outline of Hamburger and Hamilton(10), by observation of the embryos *in vivo* under 24 power magnification at the time of inoculation. Such *in vivo* observations yielded results, which, although not strictly accurate for the stage of any individual embryo, were sufficiently accurate to estimate the average stage of the group as a whole. *Examination of embryos for abnormalities.* The inoculated embryos were examined daily *in vivo* for abnormalities or at specified intervals were removed from the eggs, fixed, cleared and stained(5) for examination *in toto* under 24 power magnification. *Immune Sera.* One lot of chicken hyperimmune serum, specific for the PR8 strain of influenza A was supplied by Dr. Herald Cox of Lederle Laboratories and another by Dr. H. H. Shear of Veterans Administration Hospital, Long Beach, California. Hemagglutination-inhibition titers of the sera were 1-2048 and 1-1280 respectively. Hyperimmune serum to Newcastle disease virus, used for control injections, was prepared in chickens in this laboratory and had a hemagglutination inhibition titer of 1-2048. All sera were inactivated at 56°C for 30 minutes before use.

Results. Following procedures successfully employed with Newcastle disease virus for producing a high percentage of gross defects in early chick embryos(6) preliminary studies were made in eggs of 48 hours incubation inoculated with $10^{7.2}$ ID_{50} of influenza A virus. Control inoculations consisted of normal allantoic fluid. Of 37 embryos receiving influenza A, 29 were alive at 24 hours after inoculation and 21 of these showed specific de-

* This investigation was supported by research grant from Microbiological Institute of the Public Health Service.

fects of the lens and/or otocyst, and less frequently the neural tube. The defects consisted of retardation, microplasia or complete absence of the organ. All the embryos showing specific organ defects also showed the characteristic twisted axis and/or collapsed encephalon observed by others. Of 37 embryos receiving control inoculations, only one embryo showed any defect, this one exhibiting a mild flattening of the mesencephalon. Fig. 1 shows a normal embryo of 72 hours incubation, and Fig. 2 illustrates the characteristic appearance of a 72 hour embryo inoculated 24 hours previously with influenza A virus. Few embryos survived longer than 24 hours after inoculation of such large doses of virus.

Further experiments showed that different concentrations of virus in embryos of slightly varying developmental stages had a marked effect on the appearance of specific organ defects. Results are summarized in Table I.

Microscopic examination of serial sections of embryos showing specific organ defects, confirmed the observations on the embryos *in toto*. Lens and otocysts were absent or retarded and the cells of such tissues suffered severe damage.

FIG. 2. Characteristic appearance of embryo of 72 hr incubation inoculated 24 hr previously with a high titer of influenza A virus. Note twisted axis, flattened encephalon, absence of lens and retardation of auditory vesicle.

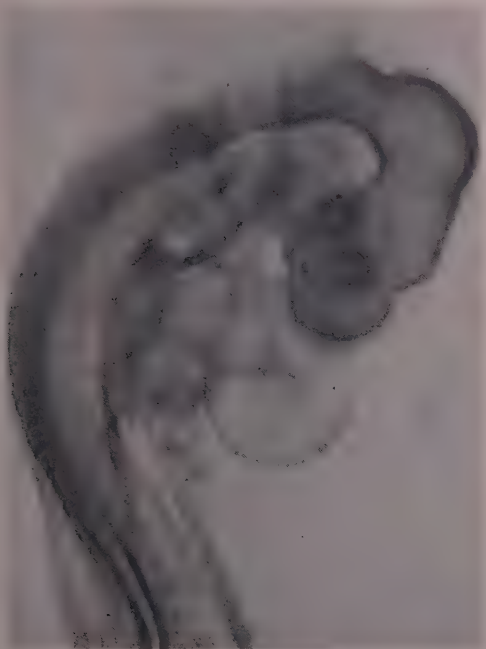

FIG. 1. Appearance of normal embryo of 72 hr incubation.

Neutralization of defects with specific immune serum. Since the gross defects of lens and otocyst produced by the influenza virus closely resembled those observed after infection with Newcastle disease virus, a check was made to rule out chance admixture of the two viruses in laboratory usage. Two lots of influenza A hyperimmune serum obtained from separate sources outside our own laboratory gave essentially the same results. Two portions of influenza virus suspension containing $10^{6.7}$ ID_{50} per 0.05 ml were mixed with equal amounts of influenza antiserum in 1-10 dilution and NDV antiserum in 1-10 dilution respectively. After 20 minutes at room temperature each was inoculated in 0.1 ml amounts into respective groups of embryos of 48 hours incubation. As an additional con-

TABLE I. Effectiveness of Different Amounts of Influenza A Virus Inoculated at Slightly Different Developmental Stages in Producing Defects in Early Chick Embryos.

Hr of incubation at inoculation	Mean developmental stage	ID_{50} inoculated	Total embryos inoculated	24 hr after inoculation: Total alive	24 hr after inoculation: Survivors with lens or otocyst defects	24 hr after inoculation: Survivors with axis twists or flattened encephalon	48 hr after inoculation: Total alive	48 hr after inoculation: Survivors with lens or otocyst defects	48 hr after inoculation: Survivors with axis twists or flattened encephalon
48	12.5	$10^{6.7}$	25	15*	15	15			
48	12.5	$10^{4.7}$	25	23	0	3 (mild)	13*	8	12
60	14.2	$10^{6.7}$	29	18*	7	10			
60	14.2	$10^{4.7}$	24	24	0	4 (mild)	19*	4	17

* Harvested at this time.

trol 2 portions of NDV virus suspension containing 10^7 ID_{50} per 0.05 ml were mixed with the influenza A and the NDV antiserum respectively and inoculated into 48 hour embryos of the same incubation setting. Results clearly show that defects of the lens and otocyst as well as the axis twists and flattened encephalon were prevented by the influenza A hyperimmune chicken serum, whereas the NDV hyperimmune chicken serum had no such effect. Moreover, the NDV hyperimmune serum neutralized the teratogenic action of the NDV, whereas the influenza A hyperimmune serum did not.

Discussion. It has been repeatedly observed that the developmental stage of the embryo at the time of exposure is an important factor in determining the type of defect produced by a teratogenic agent. In analyzing teratogenic effects in chick embryos it is necessary to take into account exact developmental stages rather than hours of incubation, since rapidity of development varies markedly depending on such factors as temperature and time of egg storage, differences in incubating conditions, genetic variation, etc. Previous studies with Newcastle disease virus inoculated in early chick embryos at different stages confirmed its similarity to other teratogenic agents in that organs in early stages of differentiation were most susceptible to its teratogenic effects(7). Although small virus inocula resulted in specific tissue damage observable on microscopic examination, gross defects were consistently observed only after inoculation of large amounts of virus. Thus the use of large inocula provides a practical tool for preliminary determination of teratogenic specificity. It is of interest that influenza A, inoculated under similar conditions, showed similar effects to those of NDV upon organs differentiating from the body ectoderm, yet also produced characteristic defects different from those observed with NDV, *i.e.*, the severe axis twists and flattened encephalon. Such marked axis twists and flattened encephalon have occasionally been observed with NDV but not as a consistently characteristic effect. Further studies of the teratogenic effects of influenza A at various stages and in varying dilutions would be of interest.

Summary. The PR8 strain of influenza A virus was inoculated in high titer in chick embryos which were in stages of development ranging from 10 to 15 by Hamburger and Hamilton staging criteria (approximately 48 hours incubation time). Such inoculations produced specific organ defects, particularly noted in the lens and auditory vesicles, in addition to previously reported axis twists and flattening of the encephalon. Embryos inoculated at slightly later stages with more dilute virus also developed axis twists and flattened encephalon, but were much less consistent in their production of defects of the lens and otocysts within the time limits of the experiment. Specific hyperimmune serum to influenza A virus completely prevented all the influenza A-induced teratogenic effects, whereas other hyperimmune serum (Newcastle disease) failed to do so.

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A Broncho-Constrictor Factor in Cigarette Smoke. (22470)

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Although cigarette smoke is of a very complex chemical nature, the pharmacological effects of the smoke have been largely attributed to the nicotine and tar content. Such studies have been well summarized by Wynder and co-authors(1). The present studies are the result of a lack of published information on acute effects of smoke on the pulmonary airway.

Procedure. Cigarettes used were "king-size" popular brands. Six brands were studied. One was available as plain-tip and filter-tip types. Only the filter tip type of one other brand was available. One brand of "denicotinized" cigarette available with and without filter tip was used. Mature stock guinea pigs were etherized and the cervical spinal cord transected. The upper trachea was cannulated with a Y-cannula, connected to positive pressure artificial respirator. The thoracic cavity was opened by mid-longitudinal section of the sternum, and the cut edges were widely separated by self retaining retractors. The animal was then allowed to recover from the ether. A separate animal was used with each cigarette. The order in which animals received smoke from different brands of cigarettes, as well as lengths of the cigarettes during the test puff was randomly mixed. Four animals were used for each brand and type of cigarette, a total of 48 animals. The positive pressure artificial respirator functioned on the principle of commercially available C. F. Palmer apparatus.* A cigarette could be attached to intake of respirator with the glass adaptor tube, so that a delay of 3 to 4 seconds occurred between drawing of smoke and inflation of lungs with the smoke. The special glass Y-shaped tracheal cannula was constructed with a side arm at the level of entrance of the cannula to the trachea. This side arm was connected to a calibrated rubber membrane manometer for recording directly on the kymograph. A record of the tracheal pressure during each cycle of inflation of lungs was thereby obtained. Emptying of lungs was passive and due only to the elasticity of lungs. The functional dead space in the apparatus was measured directly by filling the apparatus with mercury and found to be 65 ml. All cigarettes were smoked to test lengths by mechanical Robot-Smoker so that each minute it took a single 10 ml puff of 2-second duration on the cigarette.

Results. The nature of the control inflation pressure curve obtained on guinea pigs was very uniform between animals. Fig. 1 shows this typical type of control inflation curve and the effect of a single 10 ml respirator puff of cigarette smoke on the nature of the tracheal pressure curve. The different quantitative responses obtained with the various cigarette lengths studied is also presented in the Figure. The predominant effects of cigarette smoke on pulmonary inflation pressure are a more rapid increase in inflation pressure in the middle of the cycle, and a fall in inflation pressure before the end of inflation cycle. This type of effect is interpreted as being the result of a broncho-constrictor

* Ideal Respirator, available through C. F. Palmer, Ltd., London, England.

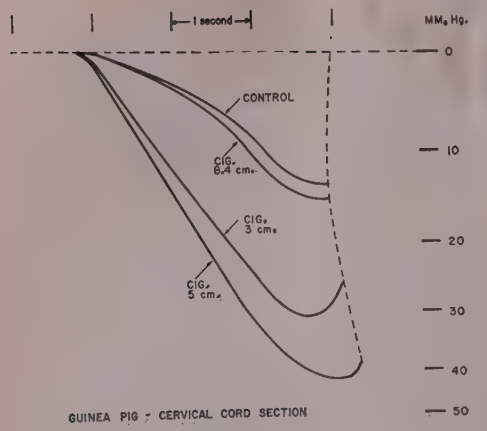

FIG. 1. Tracheal pressure curves during inflation of intact guinea pig lungs before and during response to cigarette smoke.

action of the cigarette smoke. Evidence for this interpretation of the curves is presented in the discussion.

Under conditions of a known pressure and volume of the respirator at beginning and end of each inflation cycle, Boyles Law (PV = K) may be used to calculate the actual volume of air from the pump which inflated the lungs, as a result of each pump stroke. This volume is the tidal volume. A standard graph was prepared from which any tracheal pressure value could be recorded as a given tidal volume when the respirator was set to deliver a 10 ml stroke at 9 strokes per minute. Fig. 2 consists of graph which shows maximum effect on tidal air of a single 10 ml puff of smoke from various brands of cigarettes. The least response was obtained when the cigarette length was 8.4 cm, but individual brands varied quantitatively. The greatest response was obtained when the cigarette was 5 cm long except for the "denicotinized" brand which uniformly produced less effect than other brands. Smoke from the 3 cm cigarette showed less effect than that which occurred at 5 cm length of cigarette. The Figure shows that presence of a filter tip did not significantly alter the response in the lung as compared to cigarettes with a plain tip.

Eight additional experiments showed that the lung response to cigarette smoke is not abolished by atropine. Fig. 3 shows atropine blockade of the pulmonary response to both ACh and nicotine, and failure of atropine to block the pulmonary response to cigarette smoke. Four additional experiments uniformly demonstrated that inhalation of amyl nitrite rapidly abolishes the pulmonary response to cigarette smoke.

Discussion. With artificial, positive pressure respiration under conditions of a constant stroke volume and rate, an increase above the control level in inflation pressure, measured at the tracheal level, must be interpreted as the result of a hindrance to inflation of the lung. Hindrance to inflation of lungs could be brought about either by a decrease in the caliber of bronchioles resulting from mucus secretion or bronchiolar constriction or by increase in extrabronchiolar resistance. Enhanced extrabronchiolar resistance will result in a rapid initial increase in tracheal pressure at beginning of the cycle but no decline in tracheal pressure would be expected to occur before the end of the inflation cycle. A decrease in caliber of bronchioles would likewise result in a rapid initial rise in tracheal pressure at the beginning of the inflation cycle and would, in addition, result in a fall in tracheal pressure before the end of the inflation cycle. This terminal fall in inflation pres-

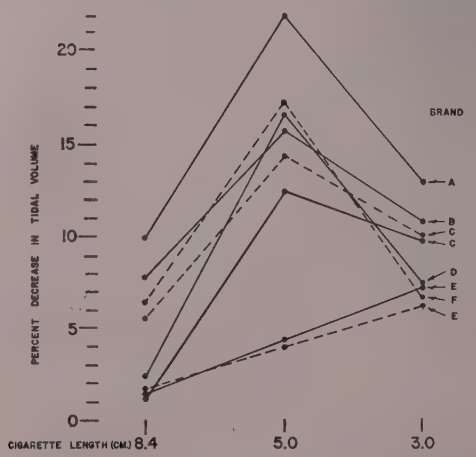

FIG. 2. Influence of cigarette smoke on tidal volume of guinea pig lungs. Each curve represents average effect on 4 guinea pigs. Filter-tip types of cigarettes are indicated by broken lines, and regular tip types of cigarettes are indicated by solid lines. Brand "E" is "denicotinized" brand.

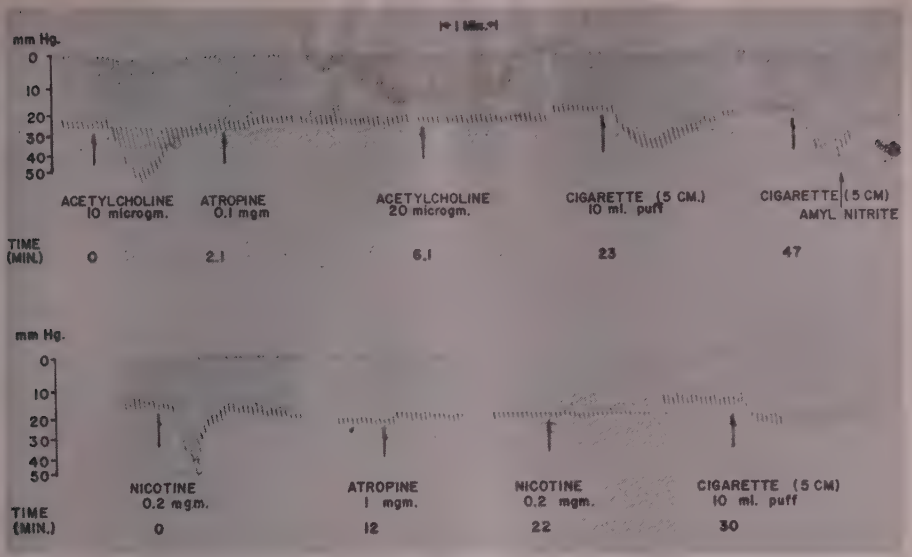

FIG. 3. Two experiments demonstrating influence of atropinization on lung inflation pressure response to acetylcholine, nicotine, cigarette smoke and amyl nitrite. All compounds were given by intracardiac injection (in the right ventricle) except the cigarette smoke and amyl nitrite which were administered by inhalation through artificial respirator.

sure near the end of the cycle would represent a "bleed-off" effect of air passing from trachea through a narrowed bronchiolar airway into the alveoli. Our experiments indicate, therefore, that the response to cigarette smoke is due to a narrowed caliber of the bronchioles. Since response to cigarette smoke appears within 10 to 20 seconds, it is unlikely that this response is due to increased mucous secretions. The rapid response can be accounted for by active bronchiolar constrictor mechanism. The failure to abolish the response to smoke by atropinization as compared to its rapid abolition by amyl nitrite indicates that the response is an atcive broncho-constrictor action which is not due to nicotine.

Reports are available concerning a subjective and edema producing irritant component of cigarette smoke(2). The irritant effects can be only partially accounted for on the basis of nicotine content of smoke. Furthermore, the tars of smoke which have been washed free of water soluble components do not produce acute irritant effects. Effects on the pulmonary airway observed could result from a non-specific irritant in the smoke. The rapid onset of effect from smoke as well as its rapid and complete abolition following inhalation of amyl nitrite indicate that airway edema is not a predominant cause of airway hindrance.

The present experiments indicate that "denicotinized" brand of cigarettes contained less of the agent responsible for the airway hindrance than does the conventional cigarettes. However, the experiments also indicate that the nicotine component is not the active agent. The other alkaloids present in tobacco (primarily nornicotine and anabasine) have some pharmacological properties similar to those of nicotine but have not been extensively studied. Tobacco of "denicotinized" brand of cigarettes may contain only minimal amounts of other constituents besides nicotine.

Our experiments uniformly showed that the greatest effect on the lung of the guinea pig occurred when the cigarette had been "robot smoked" to 5 cm. The least effect occurred when the cigarette was nearly full length (8.4 cm). This indicates that the unsmoked portion of full length cigarette acted in an efficient filtering capacity and withheld the

broncho-constrictor agent from the smoke. As the cigarette became shorter (5 cm), the retained (and possibly concentrated) broncho-constrictor agent may be volatilized by heat of the burning tip of the cigarette so that it is again carried along in the smoke. As the cigarette becomes still shorter (3 ml) an insufficient length of unburned tobacco may remain to act as an efficient filter.

Summary and conclusions. Experiments on intact guinea pigs indicate that cigarette smoke contains a broncho-constrictor agent. The intensity of the broncho-constrictor response is different with different lengths of cigarettes. Evidence is presented which indicates that the active agent in the smoke is not nicotine. Experiments using 2 brands of filter-tipped cigarettes indicated that the filter-tip did not influence the response of the lung to the cigarette smoke. The single brand of "denicotinized" cigarettes showed significantly less total broncho-constrictor effect than did the conventional cigarettes.

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Hypoglycemic Action of Orinase.* Effect on Output of Glucose by Liver. (22471)

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Bondy(1) demonstrated in humans that during postabsorption the output of glucose from the liver into the hepatic vein is in continuous state of flux, its level varying grossly and unpredictably from moment to moment. Bondy employed angio-catheterization of the hepatic vein, a method precluding sufficiently free flow of blood for precise short-interval timing required for methodology presently under consideration. We have explored these aperiodic fluctuations in normal dogs by exposing hepatic veins and directly needling the same for blood-glucose determinations at 1-minute and at 15-second intervals(2). When these readings are plotted, they assume varying configurations by their total irregularity both in rhythm and in magnitude. When similar readings are made on blood from the femoral artery, the same irregular glucose fluctuations are observed peripherally(2). Circulation time from the hepatic vein to the femoral artery was performed with fluorescine under a Wood lamp. Using this determination, one can readily identify the peripheral femoral arterial fluctuations with those emanating from liver. Irregular configuration of the hepatic undulations makes possible this peripheral identification (Fig. 1a). The original hepatic fluctuations are grossly reflected at lower over-all glucose levels throughout the main arterial tree of all 4 extremities in wavelengths of 2 to 7 minutes. When these relatively long "undulations" at both points are broken down into their structural components by taking glucose readings at 15-second intervals, one notes a markedly exaggerated variation from reading to reading, which is registered as a series of sharp flings, up and down (Fig. 1b). These may aptly be designated as "oscillations," each complete "oscillation" taking approximately 30 seconds. The

* Orinase-Upjohn (1 butyl-3p-tolylsulfonylurea) supplied by Upjohn Co. through courtesy of Dr. C. J. O'Donovan. Glucagon supplied by Eli Lilly Co. through courtesy of Drs. F. B. Peck and W. R. Kirtley. The authors are also indebted to Agnes Dann for technical assistance in 2000 glucose determinations.

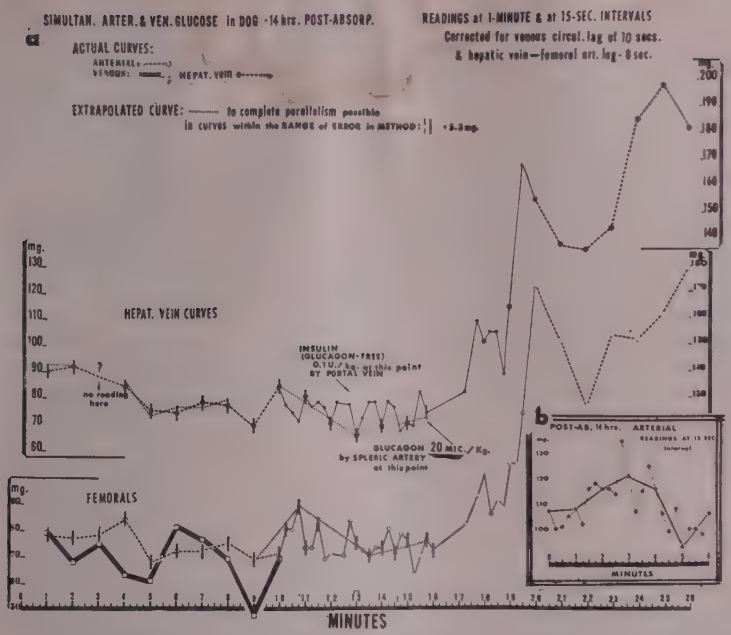

FIG. 1. a. Note parallelism of "undulations" within range of error. Component "oscillations" lose identifying configurational characteristics by passage through cardio-pulmonary circuit. b. Note wide sharp flings of "oscillations," readings at 15-sec., components of "undulations."

consistent presence of these sharp "oscillations" in hepatic venous blood throughout the period of postabsorption would suggest that during all intervals between absorption of nutrients, glucose is constantly discharged from the liver(2). At these times, however, the release occurs in sharp brief aperiodic spurts. The writers have found evidence to suggest that these aperiodic spurts of glucose from the liver are probably of glucagon origin and that they perform the physiologic function of triggering insulin into production and action during postabsorption much as does ingested glucose during the stage of absorption(3). Such fluctuations in the peripheral arteries may safely be presumed to reflect the degree and character of output of glucose by the liver into the hepatic vein. A diminished output of glucose by the liver would be anticipated to register itself during postabsorption not only as a lowered over-all arterial glucose level but also in a diminution or even a complete obliteration of the more gross undulations normally exhibited in the peripheral arteries, just as occurs after hepatectomy(2).

These principles have been applied to a study of the character of glucose output by the liver in dogs which have been subjected to intensive medication with the aryl-sulfonylurea, Orinase-Upjohn (1 butyl-3p-tolylsulfonylurea).

Procedure. For a control period of 10 days, normal dogs were fed a liberal mixed diet which included 1 to 2 lbs of beef liver daily. They were then anaesthetized (14 hours postabsorptively) with Sod.-pento-barbital by vein and heavily ergotaminized with dihydroergotamine tartrate (0.5 mg/kilo by vein). Control glucose† curves were established simultaneously on arterial and venous blood from a single extremity, the venous circulatory lag having been predetermined by an arterio-venous fluorescine circulation time

† Somogyi-Nelson(4) macro method in duplicate. Mercury-standardized pipettes. Standard error in methodology 5.3 mg/100 ml. Hematocrits followed throughout all procedures.

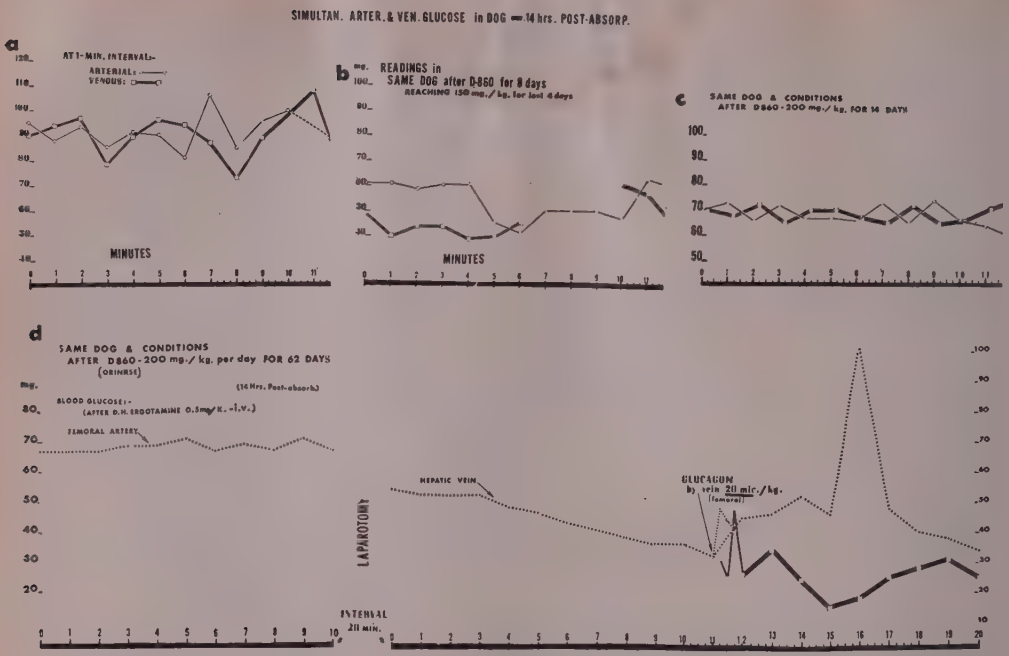

FIG. 2. Fine lines represent arterial curves. Disregard venous curves (heavy) which are irrelevant to present consideration. Glucagon I.V. at the end of curve 2c (not recorded) reestablishes arterial curve 2a in exaggerated form. Note return of hepatic undulations in 2d by glucagon I.V.

(Fig. 2a). Venous readings were ultimately found to be irrelevant to the problem at hand and although recorded are not considered. 1. One animal received Orinase orally for 8 days in progressively increasing dosage. Original blood sampling procedure was then repeated (Fig. 2b). 2. For the next 14 days the same dog was given the drug (200 mg/kilo/day), whereupon the initial sampling procedure was repeated (Fig. 2c). On completion of these readings, glucagon (20 μg/kilo) was given by vein (not recorded in Fig. 2c). 3. Thereafter, the same dosage of drug was continued for 48 days (total period of intensive drug administration being 70 days). After peripheral arterial blood sampling, the animal was laparotomized for direct sampling from the hepatic vein (Fig. 2d). The dog was then sacrificed, specimens from pancreas, adrenals, thyroid, liver and kidneys having been removed previously from the living animal for histologic examination. 4. A second dog was studied in similar manner to determine the acute effects of Orinase, the sodium salt of the drug being administered by vein in dosage of 100 mg/kilo (in 2 minutes). One hour after administration, femoral arterial blood was sampled at 1-minute intervals (Fig. 3a). Immediately thereafter, the animal received by vein in zero time 100 mg of the drug together with glucagon (10 μg/kilo), the mixture having been allowed to stand at 37°C for 1 hour. The 1-minute blood sampling was resumed. A second dose of glucagon (10 μg/kilo) was then administered by vein and the blood sampling continued. 5. For the next 14 days the drug was withheld. The animal was then again heavily ergotaminized and laparotomized (Fig. 3b) to obtain hepatic venous blood samples. Five ml of blood was withdrawn from the hepatic vein, evenly and steadily with stop-watch precision (1 ml/min.) and totally quantitated for its glucose content (Fig. 3b). The drug (150 mg/kilo) was then administered by vein over 2 minutes time. Ten minutes later, a second 5-minute glucose output rate by the liver (mg/100 ml of blood) was determined. Shortly

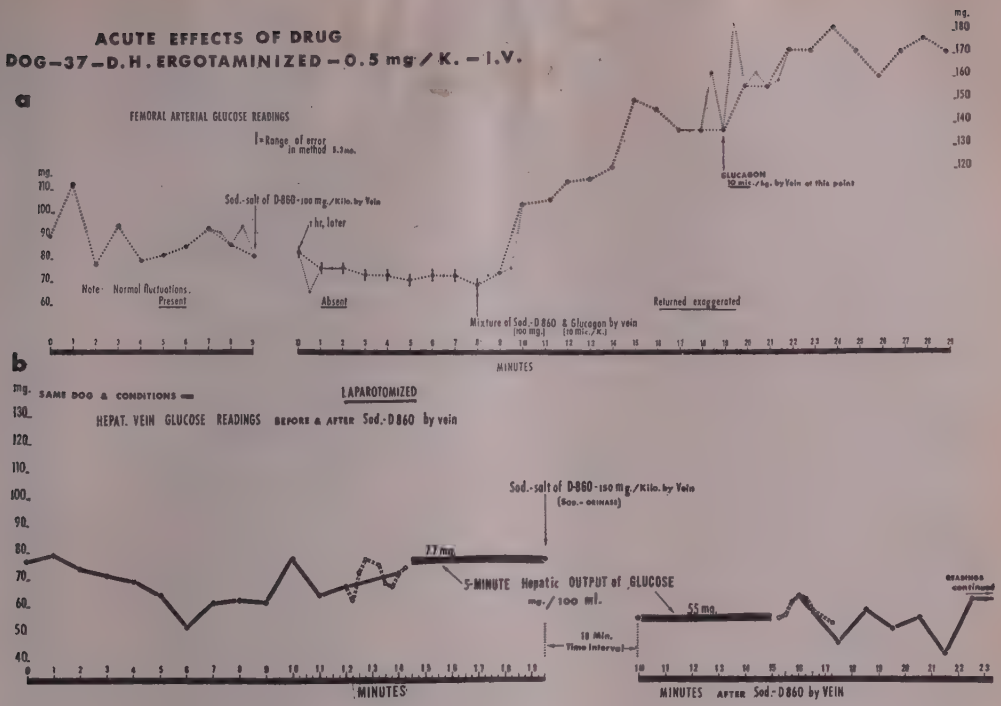

FIG. 3.

thereafter glucagon (5 μg/kilo) was given by vein in zero time (Fig. 4) and 10 minutes later a second dose of glucagon (15 μg/kilo) was administered. Shortly thereafter the animal was sacrificed for tissue examination as in the first dog.

Findings. 1. Fig. 2 exhibits a fall in mean glucose level and a progressive loss of normal peripheral arterial undulations under the influence of the sulfonylurea. After 14 days of oral exposure to the drug, arterial undulations outside the range of error have completely disappeared (Fig. 2c). These findings suggest that during postabsorption the character of delivery of glucose from the liver as well as its rate of output by that organ have changed as a result of the drug. The dose of glucagon (20 μg/kilo) which was given by vein shortly after completion of the readings in Fig. 2c resulted in a prompt re-establishment of the pre-drug arterial undulations but in markedly exaggerated form (not included in this Fig.). Accordingly, even after 14 days of intensive exposure to the drug, glycogen is still abundantly available in the liver for this exaggerated phosphorylative glycogenolysis.

2. After 70 days exposure in large dosage (200 mg/kilo, over 14 times the average daily therapeutic dose in humans), normal hepatic venous and peripheral arterial glucose fluctuations have been completely obliterated (Fig. 2d). They are, however, still capable of immediate re-establishment by a dose of glucagon by vein. Presumably the functional integrity of the phosphorylase enzyme systems I and II has in no wise been compromised (or, at least, irreversibly so) by the drug. The animal lost weight despite heavy ingestion of food. For the last 5 days before sacrificing, he showed considerable ataxia on walking and seizing food. The pancreas (including alpha-beta cell differential count), the adrenals, thyroid, liver and kidneys were normal to histological examination.

3. Orinase given by vein acts very promptly to produce changes in glucose output by the liver. Within one hour after exposure (Fig.

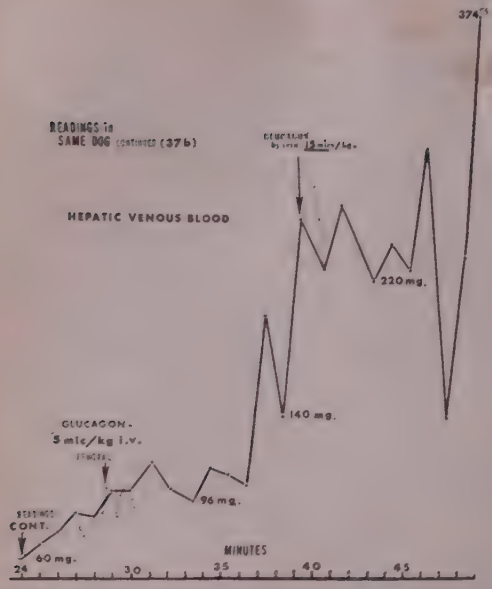

FIG. 4. Same dog as in Fig. 3. Note escape from hypoglycemia between 24th and 29th min., greatly intensified by a small dose of glucagon I.V.

3a), there developed both a fall in blood-glucose level and complete loss of normal pre-drug peripheral arterial undulations. When a mixture of the drug with glucagon was given by vein, there occurred no recognizable inhibitory effect by the drug on normal glycogenolytic effect of administered glucagon. Within 10 minutes time after a large dose of the drug by vein (Fig. 3b), there occurred a sharp decline in quantitative output of glucose by the liver (33%). The normal hepatic glucose undulations have been reduced somewhat in amplitude but have not been obliterated, their complete loss presumably taking place at some time between the 10- and 60-minute points. At the 10-minute point, the intrinsic glucagon mechanism of the ergotaminized animal is still functioning as is indicated by a persistence of the glucose fluctuations in hepatic venous blood and by the animal's spontaneous rebound or escape from hypoglycemia between 24th and 29th minute (Fig. 4). This escape is greatly enhanced by a small dose of glucagon (5 μg/kilo I.V.). Existent fluctuations are powerfully accentuated by this small dose of the hormone but extremely intensified (over 400%) by a second dose of 15 μg/kilo.

Discussion. The hypoglycemic effects of Orinase are evidently mediated, if not entirely, at least in large part, through its capacity to reduce output of glucose by the liver. There is quantitative evidence of reduction of such output within 10 minutes after intensive intravenous exposure to the drug. Within one hour after such administration, the normal minute-to-minute postabsorptive glucose fluctuations have completely disappeared and have been replaced by a flat glucose curve.

That the phosphorylase enzyme systems I and II are functionally intact after 70 days of intensive exposure to the drug is indicated by the fact that even a minute dose of glucagon is still capable of activating immediately and completely these systems to a state highly exaggerated over their pre-drug activity. The peripheral glucose fluctuations normally found in arterial blood are grossly wiped out by the drug. Since these directly reflect the character of phosphorylative glycogenolysis taking place in the liver (and expressed by output of glucose into the hepatic vein), the block in the chain of events must be proximal to the phosphorylase systems I and II. Any possible adrenal medullary effect on these enzyme systems had already been abolished by heavy ergotaminization.

A suppression in the production of glucagon at source may very well account for the observed inhibition of glycogenolysis. The prompt return of glycogenolysis on administration of a minute dose of glucagon suggests this concept. Our findings do not rule out the remote possibility that Orinase may obstruct the function of other enzyme systems which may also be proximal to the phosphorylase systems and essential links in the glycolytic effect of glucagon. No one has, however, identified any such system, nor would the effects we have observed seem to fit the functional description of Mirsky's insulinase-insulinase-inhibitor systems(5,6).

The tendency of the normal animal and of the clinical diabetic to become more sensitive to glucagon after exposure to the drug fits better with a reasoning that the drug has

caused an absolute deficiency of glucagon itself, a suppression of the hormone at source, wherever this may be. An absolute deficiency of a hormone is frequently reflected by an increased sensitivity to that hormone(7). The exaggerated physiologic response to a small dose of glucagon fits in with an absolute deficit of this hormone produced by the drug. We have found by contrast that exposure to the drug causes no significant change in the body's responsiveness to insulin. This is also in contrast with the improvement in insulin sensitivity observed in obese diabetics after adequate dietary treatment alone(7).

The speed with which the glucose lowering effect of the drug is established (10 minutes) and reversed suggests reversible interplay of enzyme systems. The anatomical integrity of the alpha cells after 70 days of intensive exposure does not support the original claim of the German workers that the drug functions by destruction of the alpha cells.

Conclusions. 1. A simple method for gauging changes in the output of glucose by the liver has been described. Throughout postabsorption, there are present in hepatic venous blood aperiodic fluctuations in glucose which are reflected throughout the major peripheral arterial tree. Under the influence of the drug, these fluctuations at both sites promptly disappear. The quantitated changing rate of output of glucose by the liver after Orinase (33% fall in 10 minutes) is drastic. A small dose of glucagon by vein immediately reverses this trend with a return in exaggerated form of the normal postabsorptive fluctuations. As a corollary one may infer that long use of the drug does not impair liver glycogen deposition. 2. It is suggested that the site of action of Orinase is proximal to the phosphorylase enzyme systems and takes the form of a suppression of glucagon production at source with a resulting reduction of glucose output by the liver. After 70 days of intensive exposure to the drug in the normal dog, acute signs and symptoms of toxicity are present but no histopathologic changes in the liver, kidneys, thyroid, adrenals or pancreas (including alpha-beta cell ratios). 3. No evidence was found to suggest that the drug works by destruction of the alpha cells of the pancreas.

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Pulmonary Vascular Changes in the Mouse During Anaphylactic Shock.* (22472)

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Discussions of anaphylaxis generally emphasize the differences in reactions in various species of animals. However, Burrage and Irwin, by direct microscopic observation of the living pulmonary circulation, have shown a marked similarity in changes occurring during anaphylaxis in the rabbit and the guinea pig(1). They noted pulmonary constriction, clumping of red cells and embolization in both species. This article will describe pulmonary vascular changes in the mouse during anaphylactic shock. The methods employed in observing pulmonary circulation were essentially the same as those of Burrage and Irwin(1).

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TABLE I. Pulmonary Vascular Reactions in Pertussis-Inoculated Horse Serum-Sensitized Mice after Challenge Doses of Horse Serum.

Mice sensitized with	Mice challenged with	No. of mice reacting* /Total No. challenged	% reacting	No. of mice with emboli /Total No. reacting	% showing emboli
.1 ml pertussis vaccine + .05 ml horse serum, i.p.	.2 ml horse serum, i.v.	37/53	70	19/37	51
.1 ml pertussis vaccine, i.p.	*Idem*	0/15	0	0	0

* Arteriolar constriction, clumping of red cells, or embolization.

Materials and methods. Female mice† weighing 17 to 25 g were sensitized by intraperitoneal injection of 0.1 ml of *Hemophilus pertussis* vaccine and 0.05 ml of horse serum. Challenge doses of horse serum (0.2 ml) were administered 10 to 20 days later via the intravenous route. Ability of *H. pertussis* to increase sensitivity of mice to active or passive anaphylaxis has been well established(2-4). The lung was exposed in the following manner: the animals were anesthetized with tail vein injections of 50-60 mg/kg of pentobarbital sodium; the 27 gauge needle employed for injecting the anesthetic was not removed from the vein and additional amounts of pentobarbital sodium were administered whenever necessary; a tracheotomy was performed and a 20 gauge needle was inserted; the needle did not fill the tracheal lumen thus allowing gases returning from the lung to escape through the opening in the trachea as well as through the animal's nose and mouth; the flow of oxygen was adjusted until respiratory movements ceased; the mouse was then turned to the left lateral position and tied down securely to the animal board; an incision was made at the level of the xiphoid process extending from vertebral column to the costal angle; vessels were ligated and muscles were divided down to the thoracic cage; a small puncture was made between fourth and fifth ribs into the pleural cavity and oxygen flow was adjusted so that minimal collapse of the lung occurred; the initial puncture wound was then extended dorsally to the sacrospinalis group of muscles, and the fourth rib was ligated and excised exposing the middle lobe of the lung. The oxygen flow was then increased in order to allow the lung to expand and fill the thoracic cage. Only a minimal amount of blood should be lost in this whole procedure. Following exposure of the lung, the animal was placed on a stand and the edge of the lung was transilluminated by the quartz rod method of Knisely(5). Light from a 500 watt projection bulb was transmitted through a fused quartz rod similar to the one described by Hoerr(6). Ringer solution at 38°C flowed out from the tip of the rod and the edge of the lung rested on this stream of Ringers. Microscopic observations were made through a Leitz binocular stereoscopic microscope using a 12X lens and 12X wide field oculars. The microscope was mounted on a special adjustable stand (American Optical) and motion pictures were taken with a Cine Kodak Special camera mounted on an adjustable tripod.

Results. Pulmonary blood flow was noted before and after an intravenous challenge dose of horse serum. The data in Table I list the observed changes. Of 53 sensitized mice, 37 (70%) showed at least one of the following phenomena after the challenge dose of horse serum: (a) sludge (clumping of the red cells); (b) arteriolar constriction; and (c) embolization. Embolization occurred in 19 (51%) of the reacting mice. In most cases the emboli were small and multiple, but in 5 instances a large single embolus which filled the lumen of a 50-150 mμ pulmonary arteriole was seen to shoot by. We were fortunate enough to obtain a film of one of these large emboli. Of 15 non-sensitized mice, *i.e.* mice injected with only pertussis vaccine, none showed any of the changes which were observed in sensitized animals after challenge

† From Tumblebrook and Carworth Farms.

doses of horse serum. It seems unlikely that the emboli observed in sensitized mice were air bubbles. Air emboli were observed in mice injected via the tail veins with saline containing air bubbles. Air emboli have thick, clearly visible refractile outer boundaries. In contrast, the outer edge of the embolus seen in anaphylactic animals was not at all distinct. In addition, air emboli were visible in the pulmonary circulation immediately after the injection of an air bubble into the tail vein. The emboli in anaphylactic mice did not appear until a few minutes after the challenge dose of antigen had been administered.

It should be mentioned that in the majority of cases administration of challenge doses of horse serum was not followed by death of the animal. Vasoconstriction, embolization and sludge were observed, but conditions returned to normal in a few minutes. In contrast, challenge doses of horse serum invariably killed 80 to 100% of pertussis-inoculated horse serum sensitized mice which were not subjected to the procedures of anesthesia, exposure of the lung and oxygen insufflation. It seems likely that the combination of anesthetic and oxygen had a protective effect in mouse anaphylaxis.

Discussion. Our observations of pulmonary vascular changes in mice during anaphylactic shock are very similar to the observations in rabbits and guinea pigs reported by Burrage and Irwin(1). Our results are also consistent with those of McMaster and Kruse(7) who described vascular changes in the ears of mice during anaphylaxis. The latter noted vascular spasm, clumping of cells and plasma skimming. It is possible that the pulmonary phenomena observed during mouse anaphylaxis account for the symptoms observed in the intact animal, namely cyanosis and respiratory embarrassment. At present the nature of the pulmonary emboli which have been seen is unknown. Our current hypothesis is that they are clumps of leucocytes sticking to an antigen-antibody precipitate.

Summary. Arteriolar constriction, clumping of red cells, and embolization have been noted in the pulmonary circulation of mice during anaphylactic shock.

We are indebted to Dr. Melvin H. Knisely for his encouragement and his many helpful suggestions.

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Sustained Propagation of Sarcoma 180 in Tissue Culture.* (22473)

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The delineation of the specific amino acid, salt and vitamin requirements of a mouse fibroblast (strain L) and of a human carcinoma (strain HeLa)(1-6) has permitted the continuous propagation of these cells in a medium containing only demonstrably essential growth factors, in which the omission of a single amino acid or vitamin, of glucose or serum protein, resulted in the cessation of growth and cell death. Eagle also has reported the successful isolation and propaga-

* This investigation was supported in part by research grants C-937 and C-2782, from National Cancer Institute of N.I.H., U.S. Public Health Service.

tion of a human epidermoid carcinoma (strain KB) directly from a biopsy specimen in these media(7). Several other normal and malignant human and animal cells also have been cultured directly from surgical material into media embodying these same minimal requirements supplemented with 10 to 20% whole serum, and a number of previously established cell lines have been similarly propagated(8-10). The purpose of the present report is to record the isolation and continuous propagation of Sarcoma 180 (S-180) in these ("HeLa") media directly from biopsy specimens.

Experimental. The medium, prepared as described by Eagle(1-7), contains 13 essential amino acids, 7 essential vitamins, glucose and salts, each in the concentration necessary for optimal growth of the HeLa cell. All experiments were done in duplicate series. The medium used in one series was supplemented with 10% human serum, while parallel experiments were done in media prepared with 10% horse serum. Similarly, the effect of various supplements to the basal substrate was studied in replicate experiments employing both media. Biopsy specimens of S-180 maintained in CFW mice† were minced with sterile scissors and suspended in 15-25 ml of complete growth medium. Trypsin,‡ 0.25% final concentration was added and the suspension incubated at 37°C on a "Magnimix" stirrer (adjusted to produce gentle agitation) to disperse the cells, as described by Youngner(11). The turbid supernatants containing dispersed cells were removed at 15-20 min intervals and stored in an ice bath. The bits of tissue remaining in the original flasks were resuspended in fresh media, and the trypsin-dispersing process repeated several times until no gross particles of tissue remained. The supernatants were pooled, centrifuged at 500-600 rpm for 3-5 min and washed 3 times with 10-15 ml of media to remove the trypsin. The washed cells were resuspended in media without serum and aliquots of the resulting heavy suspension were inoculated into 3 ml of complete medium in culture flasks(1,12) with a surface area of approximately 15 sq cm and incubated (stationary) at 37°C. For quantitative experiments, the washed cells were enumerated directly by haemocytometer counts, diluted appropriately and cultured in a similar manner.

TABLE I. Rate of Growth of S-180 in "HeLa" Medium.

Cell count × 10^4,* after hours incubation								
0†	24	48	72	96	120	144	168	192
6.0	6.6	14.7	33.0	60.2	105.4	116.0	151.3	203.5

Age of inoculum (days)	Cell counts × 10^4* Hr incubation		
	0	48	96
5	6	14.7	60.2
10	9	5.5	44.5
15	6	6.2	25.0

* Mean values of 2 experiments in replicate.
† Inoculum prepared from 5-day-old culture.

Results. The original cultures initiated in this manner on 16 June, 1955 grew poorly in media prepared with human serum, even when supplemented with 2% beef embryo§ extract. The cultures in media containing horse serum grew slowly at first, and there was a definite favorable response to beef embryo extract. The media were changed after 48 hrs and at 48 hr intervals thereafter, without disturbing the cells. After 11 days, the cell layer growing on the glass in the original cultures was exposed *in situ* for 1-2 min to 0.125% trypsin‡ (final concentration) in fresh complete media, the resulting free cells and cell clumps washed and subcultured in media containing 10% horse serum, with and without 2% beef embryo extract. The rate of growth continued to increase with successive transplants only in media containing beef embryo extract until after the 4th transplant on glass; thereafter beef embryo extract was no longer required. At this time, the cells were transplanted to 1-liter Blake bottles (surface area of approximately 150 sq cm) in media containing 10% horse serum and have been so maintained since that time with transplants

† This strain of Sarcoma 180 was obtained through the courtesy of Dr. F. Homburger, Cancer Research and Cancer Control Unit, Tufts University Medical School, Boston, Mass.

‡ Difco "1:250".

§ Microbiological Associates, Bethesda, Md.

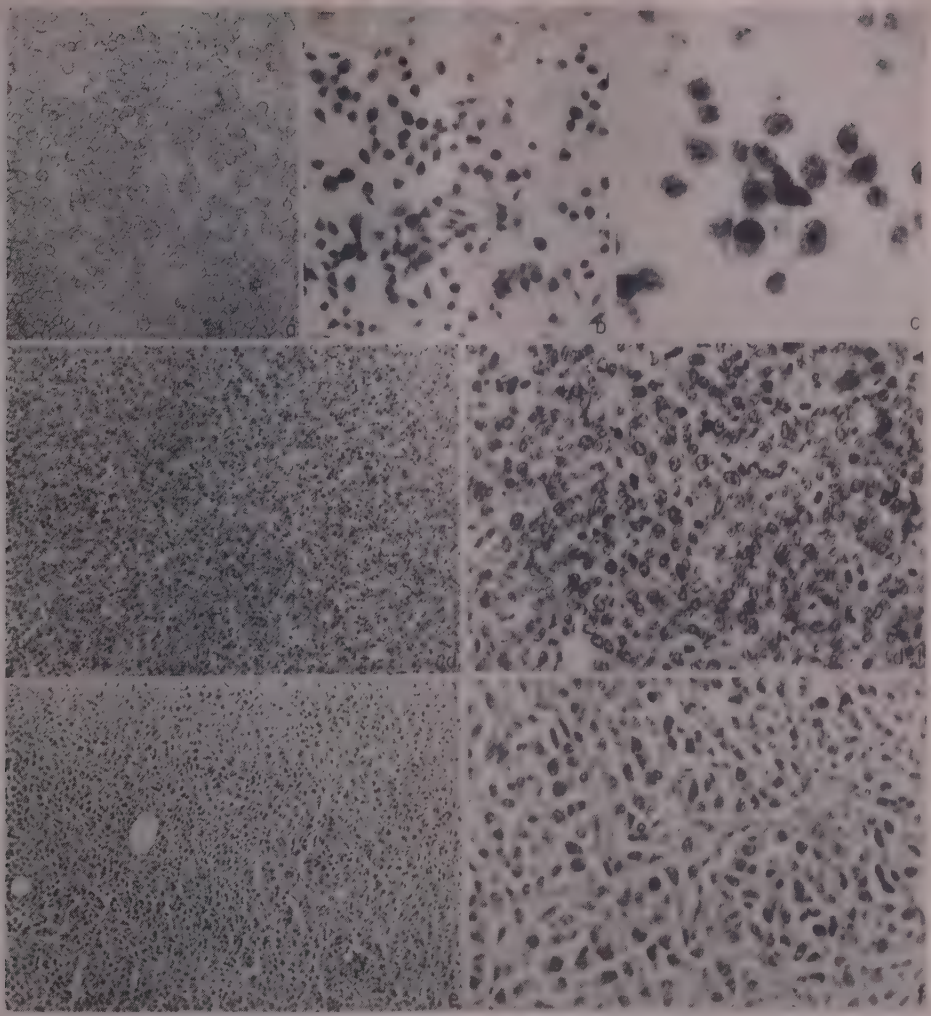

FIG. 1. (a) S-180, living, 6-day-old culture of 24th *in vitro* transplant growing *in situ* on glass. Unstained, ×137. (b) Same as (a). Collodian fixation, H & E, ×137. (c) Same as (b), ×417. (d) S-180 in CFW mice. 15-day tumor produced by usual trocar implant. H & E, ×137. (d-1) Same as (d), ×417. (e) S-180 in CFW mice. 15-day tumor produced by tissue culture inoculum of 2.5×10^5 cells in 0.1 ml media. H & E, ×137. (f) Same as (e), ×417.

at 5-7 day intervals.

The rate of growth of S-180 as determined in a typical experiment by haemocytometer counts is illustrated in Table I. The growth rate appears to have become stabilized at a generation time of *circa* 30 hrs in the logarithmic phase after 20 *in vitro* transplants, thus approximating that observed with the KB cell(7). Experiments on the effect of inoculum age on the lag and early logarithmic phase of the growth curve (Table I) indicate that the optimal time for transplantation is during the period of maximum metabolic activity. The microscopic appearance of the adherent, syncytial cell layer in living, glass-surface cultures is illustrated in Fig. 1a. The typical morphology of these cells in stained

TABLE II. Titration of S-180 Tissue Culture Inocula in CFW Mice.

Cells/inoculum*	Morbidity Tumors/Mice	%	Latent period, days Mean	Range
More than 10^5	20/20	100	3.5	3– 5
10^5	20/20	100	3.5	3– 5
10^4	16/20	80	8.6	6–13
10^3	7/20	35	19.7	16–27
10^2	2/20	10	29	29–34
10	2/20	10	29	29–34
10^{-1}	0/20	0		
Diluent control	0/20	0		
	ED_{50} data			
Expected	10/20	50	7	3–16
Observed (5×10^2 cells)	8/20	40	6.6	5–15

* Cells from 5-day-old cultures in volume of 0.1 ml subcut. in inguinal.

preparations is illustrated in Fig. 1b, c.‖

The cells obtained from trypsin-treated cultures of transplant numbers 5, 8, 17, 20, 23 and 26 after 38, 117, 165, 186, 200 and 215 days respectively *in vitro* produced typical sarcomas‖ (Fig. 1d, e, f) following incubation periods of 3-5 days when implanted subcutaneously in CFW mice. Suspensions prepared from these tissue cultures can be quantitated and titrated in mice as has been done with tumor breis or ascites cells by several investigators(13-19), and the ED_{50} computed (20). The results of a typical experiment done with the 20th transplant (after approximately 60 generations *in vitro*) are summarized in Table II. The minimal inoculum resulting in tumors in 50% of the mice implanted has been observed repeatedly to be *circa* 4.5-5.0 x 10^3 cells, about 90% of which are viable as judged by the trypan blue technic(21). The time:dose relationship characteristic of decreasing inocula also can be used to predict within reasonable limits the incubation period following implantation, and the expected morbidity:time relationship agrees reasonably well with observed values, as illustrated by the typical experiment summarized in Table II.

Implantation of replicate inocula of 1.0 x 10^5 or more cells subcutaneously in CFW mice thus far has resulted in 100% morbidity, but the tumors so induced exhibit considerable variation in size (Table III). That this variation is due at least in part to differences in the growth rate of the tumor cells in different mice is indicated by the observation that inocula of 1.0 x 10^5 or more cells produce palpable tumors in all mice so injected within 3-5 days. The host factors determining these differences are as yet undefined, although there is some suggestion that tumors induced in female CFW mice are smaller than those in male mice (Table III). It is possible that variable immunological responses in individual mice tend to select from the inoculum cells of differing growth potentials. The isolation by *in vitro* technics of HeLa cells showing differing characteristics under various conditions of growth has been demonstrated recently(22). However the tissue culture sublines derived from small and large tumors by re-isolation *in vitro* thus far

TABLE III. Variation in Size of Tumors Induced by Replicate S-180 Tissue Culture Inocula in CFW Mice.

Inoculum*	Tumors/Mice	Days	Wt tumors in mg Range	Mean
23rd *in vitro* transplant				
10^5 cells	20/20	5	3- 32	10.5
	" †	10	8- 346	137
	"	15	15-1529	246
26th *in vitro* transplant‡				
2.5×10^5 cells§	(♂) 10/10	7	30- 159	87
	(♀) " †		38- 102	63
	Total		30- 159	75
	(♂) 10/10	14	102- 522	290
	(♀) "		51- 314	170
	Total		51- 522	230
5.0×10^5 cells	(♂) 10/10	7	60- 175	108
	(♀) " †		35- 106	72
	Total		35- 175	90
	(♂) 10/10	14	143- 683	375
	(♀) " †		123- 348	248
	Total		123- 683	311

* Cells from 5-day-old cultures in vol of 0.1 ml subcut. in inguinal.

† One tumor lost before weighing.

‡ Inocula prepared from untrypsinized cultures showed similar variations in tumor size.

§ Tumors induced by this inoculum suspended in 0.4% agar-agar exhibited similar variations in size.

‖ We wish to thank Dr. J. M. Craig, Department of Pathology, The Children's Medical Center, for these histological studies.

have exhibited similar variations in the size of the resulting tumors following implantation in CFW mice.

Discussion. The use of beef embryo extract (and other supplements) in the basal medium in the original experiments was prompted by earlier observations that S-180 and a variety of other animal and human tumors initiated growth but failed to survive serial subculture in the unsupplemented medium. The survival of S-180 and a spontaneous sarcoma of Syrian hamsters(8) in supplemented media suggests that for these cells the beef embryo extract provided the factor(s) required for survival which were either unavailable or deficient in the unsupplemented medium. Fischer(23) has suggested that cells implanted in artificial substrates may require additional nutritional factors until acclimated to their foreign environment. On the other hand, the epidermoid carcinoma isolated by Eagle(7), 5 Wilm's tumors, and a teratoma isolated from biopsy specimens(8) all grew vigorously when implanted in the unsupplemented medium. Whether or not the ultimate growth of S-180 in unsupplemented media represents a biochemical adaptation to the substrate cannot be stated at the present time.

S-180 is now well established in Blake bottle cultures and at this time is in the 32nd transplant, having undergone more than 100 generations during 230 days *in vitro* in these laboratories. This cell line also is being cultivated on a large scale for commercial distribution.§ The ready induction of specific amino acid and vitamin deficiencies in S-180 as has been described for other cell lines in these media(1-4), and their reversal by restoration of the missing metabolite, suggested the use of this cell line for the assay of the cytotoxic action of carcinolytic agents in tissue culture(24). Detailed experiments on the nutrition and cytology of S-180 in these tissue cultures are now in progress.

It is evident that these *in vitro* cultures of S-180 have thus far retained the capacity to induce typical sarcomas in CFW mice. Whether or not this characteristic will be maintained indefinitely is conjectural at this time; however two strains of human epidermoid carcinomas which have been maintained in these media for a much longer period of time induce typical epidermoid carcinomas when implanted into the cheek pouch of golden hamsters(25). The time:dose relationships observed with tissue culture inocula of S-180, like those reported with epidermoid carcinomas in hamsters(25), may prove to be useful in the quantitative study of potential chemotherapeutic agents. Tissue culture cells also present advantages as source inocula for the routine propagation of S-180 in mice, since such inocula are free of extraneous bacteria and are readily quantitated. Further experiments in this direction are now in progress.

Summary. 1. A strain of Sarcoma 180 maintained by routine trocar implant passage in CFW mice has been isolated and serially propagated *in vitro* from biopsy specimens in a medium containing only the growth factors demonstrably essential for the HeLa cell and the L strain of mouse fibroblasts. The generation time in these media is *circa* 30 hrs and this cell line is now in the 32nd transplant, having undergone more than 100 generations during 230 days *in vitro*. 2. Inocula prepared from these cultures after 215 days *in vitro* have produced characteristic sarcomata within 3-5 days in 100% of the CFW mice implanted subcutaneously with 1.0×10^5 or more cells. The ED_{50} in CFW mice is *circa* $4.5\text{-}5.0 \times 10^3$ cells, and the latent period between injection and the development of palpable tumors increases with decreasing inocula.

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Rate of Displacement of Oxytocic Substances from Dyencephalon to Tuber Cinereum in Hypophysectomized Rats. (22474)

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We reported(1) an increase in concentration of oxytocic substances in the tuber of hypophysectomized rats killed 5 to 14 days after hypophysectomy. The oxytocic activity, determined by its effect on uterus *in vitro*, was approximately 8 times higher in the tuber of hypophysectomized animals than in nonhypophysectomized controls.

In the present work we have attempted to determine the rate at which this change takes place by determining the oxytocic activity of tuber extracts obtained from hypophysectomized animals killed half an hour after hypophysectomy.

Method. Fifteen experimental sets of animals were used, each one of them included the following groups: 1) nonhypophysectomized animals; 2) animals killed half an hour after hypophysectomy; 3) animals hypophysectomized after death. Hypophysectomy was performed through the base of the skull in animals anesthetized with avertin by intraperitoneal injection, the anesthesia lasting 20 minutes. All animals were anesthetized and killed in a gas chamber (death in about 3 minutes) when the effects of anesthesia had disappeared. The sets included animals of the same sex, weight, and age, each group containing 4 animals, with a total of 59 nonhypophysectomized and 67 in each one of the other groups. Separate extracts were prepared immediately after the animals were killed using equivalent amounts of tissue from the tuber. Tissue from 4 animals belonging to the same group was ground with micronized quartz sand and 0.5 ml of water (acidified to pH 3) per 200 g of rat weight was added. The extract was centrifuged at 2000 rpm for 15 minutes. The oxytocic activity was determined on a uterus of a rat in diestrus immersed in Tyrode solution prepared according to Gaddum with the addition of 2 mg of atropine per liter(2). Previously neutralized extracts were added to the bath in volumes ranging from 0.1 to 0.5 ml. The oxytocic activity of the unknowns was obtained by proper dilution and comparison with a standard solution of commercial oxytocin ("Pituitrol") containing 10 u. per milliliter.

Results. A higher oxytocic activity of the tuber region was found in animals hypophysectomized and then killed than in animals hypophysectomized after death. Only in 4

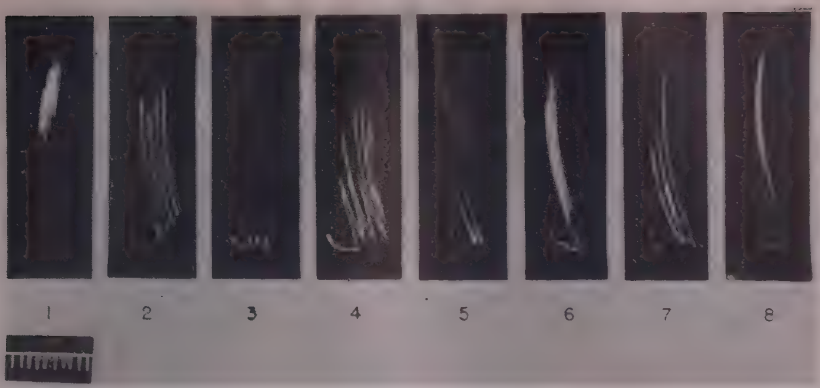

FIG. 1. Contraction of isolated uterus immersed in Tyrode solution. Additions: 1—1 mu oxytocin. 2—.2 ml extract of tuber from rats killed half hour after hypophysectomy. 3—.2 ml extract of tuber from rats hypophysectomized after death. 4—.4 ml, same as 3. 5—.1 ml, same as 2. 6—.6 ml, same as 2. 7—.6 ml, same as 3. 8—1 mu oxytocin.

animals no difference was observed in relation to those killed prior to hypophysectomy. The oxytocic activity of the tubers of animals hypophysectomized and then killed was about twice as high as those of animals hypophysectomized after death. No difference was found between the activity of the tubers of animals killed before hypophysectomy as compared to nonhypophysectomized animals, with the exception of 4 sets, in which the oxytocic activity was slightly higher in the latter.

Discussion. The increase of the oxytocic potency observed in the tuber extracts of hypophysectomized rats shows the rapidity of the change in concentration of the active principles of this hypothalamic region. Such an increase may be explained as an accumulation of active material which cannot continue in its way to the neurohypophysis, the latter representing the discharge area. This observation appears to be in agreement with previous findings showing rapid changes in the sectioned pituitary stalk(3,4) or in the median eminence (5) after application of painful stimuli.

On the other hand Rothballer(6) has reported significant changes in the distribution of the neurosecretory material of the diencephalon 4 to 6 minutes after application of a painful stimulus.

Even when painful manipulations are avoided, it is possible that the changes observed in the concentration of oxytocic substances are due to the stress preceding the extraction of the encephalon rather than to the extirpation of the hypophysis.

The preceding results support the hypothesis that the active principles of the neurohypophysis are produced in the hypothalamus and are rapidly mobilized to the discharge area.

Summary. Extracts of tuber cinereum from 67 hypophysectomized rats obtained half an hour after the operation, showed an oxytocic potency twice as high as that observed in tuber extracts of 67 animals hypophysectomized after being killed.

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Autoradiographic Study of Localization of Aurintricarboxylic Acid in Experimental Beryllium Poisoning.* (22475)

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The effectiveness of aurintricarboxylic acid (ATA) in the treatment of acute beryllium poisoning in experimental animals has been described(1,2). The therapeutic effect of this dye is ascribed to the fact that it combines readily with free beryllium ions in tissues to form a chelate and thence a stable lake which renders beryllium innocuous to tissues in which it is deposited. Histopathological studies of the principal target organs in acute beryllium poisoning have shown that ATA produces arrest of damage in liver, spleen and bone marrow followed by prompt repair of the lesions, thus allowing animals to survive a toxic dose of beryllium ($LD_{98/3\ days}$) for an indefinite period of time(3). Anatomical distribution of early lesions within an organ can be correlated with the distribution pattern of beryllium as described by Kaylor and Van Cleave, who visualized the deposition of tracer amounts of radio-beryllium in tissues of rats with the aid of autoradiograms(4). Studies of C^{14}-labeled ATA in the rat have shown a high and almost constant level of retention of this material in liver, spleen and kidney up to 3 months or more(5). The following experiment was undertaken in an attempt to visualize the simultaneous localization of beryllium and ATA in various organs of mice.

Methods. Beryllium was prepared for injection by dissolving carrier $BeSO_4$ and carrier-free Be^7Cl_2 to give an aqueous solution of pH 3.5 with a radioactivity level of about 4.3 mc/ml; the dose was 0.54 mg/kg (LD_{70}) and about 1 mc Be^7 per mouse. The ATA injection consisted of a 3% solution of ammonium salt of C^{14}-methyl-labeled ATA adjusted to pH 7 and containing approximately 0.25 mc C^{14}/ml; the dose was 200 mg/kg with 0.05 mc of C^{14} activity per mouse. Twelve young adult CF#1 female mice weighing 30 g each were divided into 3 groups of 4, receiving intravenously: (I) Be only; (II) Be, followed after one hour by ATA; (III) ATA only. Two animals of each group were sacrificed by cervical fracture at 12 and 48 hours after injection. Kidney, liver, lung and spleen were fixed in 10% neutral formalin and embedded in paraffin, and sections were cut at 15 μ. Autoradiograms of Be-containing tissues were prepared by a technic similar to that of Kaylor and Van Cleave(4), using type 506 Ansco X-ray film and 0.5 mil gold foil in contact with the sections at −10°C for 3-6 weeks. Autoradiograms of tissues containing ATA were prepared in the same manner except for the elimination of gold foil between film and section. For detection of ATA in tissues containing both Be and ATA the sections were stored until Be^7 was no longer detectable in control sections (4 half-lives) after which the sections were again autoradiographed. Finally they were stained with hematoxylin and eosin for histologic examination.

Results.† *Liver.* All of the beryllium autoradiograms showed a diffuse blackening with an occasional area of more intense activity; in no case was it possible to correlate radioactivity either with normal anatomic structures or with areas of liver damage. Failure to demonstrate a characteristic pattern in the deposition of beryllium in this organ was in contrast to the results reported by Kaylor and Van Cleave(4) who showed liver autograms with numerous punctate areas of concentration of radioactivity, corresponding with the peripheral parts of liver lobules. ATA pictures likewise failed to show a distinct pattern of localization in the liver; when ATA was given alone the liver showed a uniform degree

* Work performed under the auspices of U. S. Atomic Energy Commission.

† Illustrations are restricted to reproductions of C^{14}-ATA autograms since Be^7 autograms, although adequate for analysis, do not readily lend themselves for reproduction.

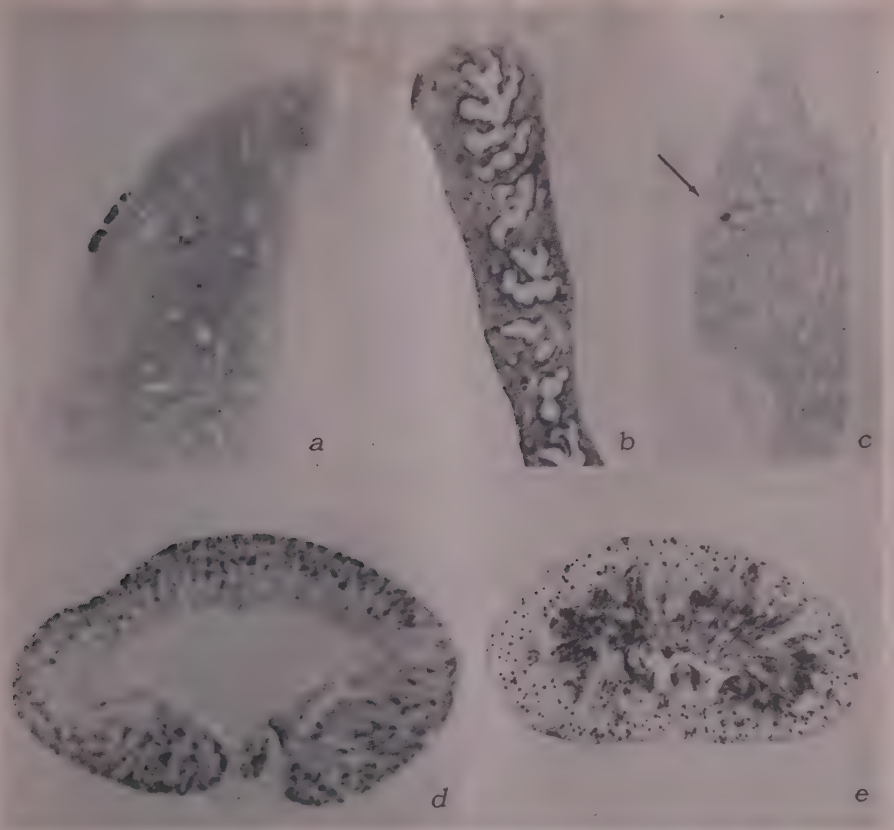

FIG. 1. Autoradiograms showing distribution of C^{14}-ATA in tissues of beryllium-poisoned mice. All magnifications 5.5 ×. (a) Liver; generally uniform distribution of ATA. Discrete areas of high concentration correspond to focal necroses of liver cells. (b) Spleen; concentration exclusively in red pulp, especially in marginal sinuses. (c) Lung; focus of intense radioactivity associated with thrombus in branch of pulmonary artery. (d) Kidney; ATA given alone; concentration in tubules in outer cortex and absence of radioactivity from medulla. (e) Kidney; animal given Be and ATA; concentration of ATA primarily in damaged glomeruli and tubules.

of blackening of the film. In contrast, 2 animals with beryllium-induced liver damage showed in addition a striking concentration of C^{14} activity as small punctate dots and streaks (Fig. 1a); these corresponded to areas of focal necrosis of liver cells.

Spleen. The beryllium and ATA autoradiograms in all groups showed an identical pattern of localization of the 2 substances in this organ; the radioactivity was confined to the red pulp, leaving the lymphatic nodules as sharply outlined pale areas. The perifollicular or marginal sinuses of the red pulp showed the greatest concentration of Be and ATA, and as a consequence they appeared as intensely dark rings (Fig. 1b).

Lung. Beryllium was demonstrable in all lungs at both intervals but it showed no specific pattern of distribution. ATA pictures showed a mild degree of diffuse darkening, sparing the lumina of bronchi, blood vessels and alveoli. In all animals and at all intervals there were many discrete small foci and thin streaks of higher radioactivity; these were associated with intrapulmonary lymphatic nodules and lymph channels. One autoradiogram, obtained from an animal 48 hours after injection of beryllium and ATA, showed an intensely dark spot of radioactivity which was found to correspond to a fresh

thrombus in a pulmonary artery (Fig. 1c). The beryllium autogram showed no corresponding increased activity in this region.

Kidney. In animals injected only with beryllium, radioactive material was deposited throughout the renal cortex but was absent in the medulla. The cortical deposition appeared in two distinct compartments of different concentration: an outer zone, showing a generally mild degree of blackening of the film, and a narrow inner zone along the cortico-medullary junction, which showed more intense blackening. When both beryllium and ATA were given, there was a shift in the beryllium radioactivity from the inner zone toward the outer zone; and instead of being evenly distributed, beryllium was concentrated in large and small streaks, some of which extended throughout the cortex. These streaks corresponded to tubular injury in the inner and outer zone of the cortex. Animals injected only with ATA showed a very characteristic concentration of radioactivity in the cortex. It appeared as little clumps and as discontinuous and slightly tortuous bands; the activity was greater in the outer than in the inner zone and corresponded to kidney tubules. There was no radioactivity in the glomeruli or in the medulla (Fig. 1d). The ATA autoradiograms of beryllium-poisoned animals showed changes in distribution with a concentration of ATA primarily in damaged tubules, including those in the medulla. Of particular interest was the kidney of an animal sacrificed at 12 hours which showed numerous punctate foci of radioactivity throughout the cortex and heavy streaks in cortex and medulla (Fig. 1e). Each punctate focus corresponded to a severely damaged glomerulus, whereas the coarse streaks represented radioactivity in injured kidney tubules with necrotic epithelium and casts in the lumina.

Comparison of overall densities of autoradiograms obtained from the three groups of mice revealed the following: In the kidney and lung of Be-injected, ATA-treated mice there was generally a higher uptake at 12 and 48 hours of both Be and ATA than in the animals receiving either alone. In the liver and spleen there appeared to be a loss of Be at the 12-hour interval as a result of ATA treatment.

Discussion. The autoradiographic visualization of the distribution of ATA and beryllium in tissue confirms earlier chemical findings and previous observations on the therapeutic effect of ATA on beryllium-induced tissue damage. In general, ATA has been found localized in areas that contained beryllium and exhibited beryllium damage. An identical pattern of localization was noted especially in the spleen, where the patterns of beryllium and of ATA distribution in the red pulp corresponded closely with areas of tissue damage noted in morphological studies(3) and with earlier histochemical observations of Aldridge *et al.*(6). In the liver and lung, where no specific areas of high concentration of either ATA or beryllium were found, it may be assumed from the uniform distribution of the two throughout these organs that there was opportunity for contact between ATA and beryllium. The linear and small focal aggregates of ATA that were correlated with lymphatics and lymphatic nodules in the lung are perhaps best explained as representing ATA that was drained from the lung by the lymphatic system, having been deposited initially in the phagocytic cells of the lung in a manner identical with the removal from the circulation of ATA by the phagocytic cells in the spleen and liver. The kidney was the only organ showing minor differences in the localization of Be and ATA when given separately. While both substances were restricted to the renal cortex, beryllium was found mainly in the inner zone while ATA was concentrated primarily in the outer zone of the cortex. When both substances were given together there was a change in the distribution of both substances and this could be readily correlated with damaged areas in the kidney. The increased concentration of ATA in the damaged tissue in kidney and liver probably was the result of aggregation of formerly evenly distributed material in the collapsed necrotic parenchyma. Potentiation of beryllium-induced kidney damage by a dose of 400 mg/kg of ATA has been described earlier(3), but it is uncertain whether and how the shifts

in localization of Be and of ATA may be related to this phenomenon. A similar potentiation of kidney damage has been observed in cadmium-poisoned rabbits treated with BAL(7). The critical importance of renal damage in animals undergoing treatment which promotes redistribution of toxic metals in the tissues warrants further investigation of the complex situation which prevails in the kidneys of animals undergoing such treatment.

Summary. The distribution of Be^7 and of C^{14}-labeled aurintricarboxylic acid (ATA) was studied autoradiographically over a period of 48 hours in the spleen, liver, lung and kidney of mice. It was found that in general beryllium and ATA were deposited in similar tissue loci. The highest simultaneous concentrations and identical patterns of deposition were seen in the red pulp of the spleen. Other organs showed reasonably good correlations in deposition of the two substances, confirming earlier therapeutic studies. An increased concentration of C^{14}-ATA was observed in areas of tissue damage produced by beryllium in the liver, kidneys and lung.

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Histochemistry XLIV. Use of Density Gradient for Isolation of Mast Cells.* (22476)

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Padawer and Gordon(1) described a differential centrifugation technic for the rapid separation of mast cells from peritoneal fluid of the rat. They flushed the peritoneal cavity with 2 or 3 ml of 0.9% NaCl, layered the cell suspension over a sucrose-gelatin-NaCl solution and centrifuged the mast cells down into the latter medium while retaining the less dense cells above the interface between the layers. Thus they were able to obtain suspensions containing more than 75% mast cells.

Effective use of density gradients for the separation of cellular particulates(*e.g.* 2-7) suggested that this principle might offer advantages in the separation of cells. The first study undertaken in this connection was the isolation of mast cells which were required for other investigations in this laboratory. Significant improvements in the method for isolation have been effected, and suspensions which were 100% pure with respect to cell type have been obtained with yields as high as 87%.

Modified procedure. Padawer and Gordon exsanguinated the rat under ether anesthesia before opening the abdomen by a mid-ventral incision. In an attempt to minimize further the contamination of the peritoneal fluid with blood, and the associated clotting which entraps many mast cells, other procedures were tested in the present study. The most effec-

* This study was supported by research grants RG 3911 and H 2028 from N.I.H., U. S. Public Health Service, and the Medical Research Fund of Graduate School, University of Minnesota.

tive proved to be the use of an electrically heated wire loop or cautery knife for the incision, which reduced the red cell count in the original suspension about 7 times. Prior exsanguination seemed to have little effect when the cautery was used, but the simplest method, and one which would retain any advantage of exsanguination while eliminating anesthesia, would seem to be decapitation, blood drainage, and cautery incision.

Another change introduced was replacement of 0.9% NaCl by Hanks' balanced salt solution(8) to provide a more physiological medium. Occasional clotting, which was still encountered in the peritoneal wash, was considerably reduced by the addition of 50 μg of heparin per ml salt solution. No effects from the heparin were noticed over a 1 hour period.

The high viscosity due to the gelatin in the medium of Padawer and Gordon made it difficult to use, and it was found that the Diodrast-sucrose medium(5,6) which avoids this difficulty renders the mast cells fragile and destroys their ability to stain metachromatically with toluidine blue. Accordingly, only sucrose was employed for the density gradient. The finding of Ottesen and Weber(6) that Diodrast-sucrose gradients require 24 hour standing at 2°C for the density to become continuous, as shown by schlieren curves recorded with an electrophoresis apparatus, was found to apply to the sucrose gradients used in the present work. It was noted that sucrose gradients could be stored in the cold room (4°C) for 2 days without noticeable diffusion. Phosphate buffer, as employed by Ottesen and Weber, was also used in the sucrose gradients. The addition of Versenate to the buffer reduced the tendency of clumping of the leucocytes and erythrocytes. Such clumping trapped mast cells and thus reduced their yield.

To observe whether convection was a disturbing influence in the centrifugation procedure carried out at room temperature (25°C), a comparison was made with the entire procedure conducted in the cold room (4°C). No advantage followed from the latter; in fact, the greater viscosity of the medium caused the mast cell layer to remain closer to the leucocyte layer and the latter retained more mast cells which resulted in poorer yields.

The pattern of centrifugation recommended by Anderson(7) was found to be advantageous, and the time and centrifugal force used by Padawer and Gordon(1) proved to be suitable for the present purpose.

The gradient tubes are set up as follows: Prepare and store in the cold (a) Versene-phosphate buffer, pH 7.4 (0.190 g disodium Versenate, Bersworth, 2.86 g $Na_2HPO_4 \cdot 12H_2O$, and 0.272 g KH_2PO_4 to 1 l. with distilled water), (b) Sucrose stock solution (53.7 g sucrose to 46.3 ml of Versene-phosphate buffer), (c) Sucrose working solutions of sp.gr. 1.200, 1.165, 1.130 (Dilute 7.95, 6.55 and 5.15 ml of stock sucrose solution respectively with Versene-phosphate buffer to final volumes of 10 ml). Carefully layer, with a minimum of mixing, 1 ml of each of the 3 sucrose working solutions in a Pyrex tube 13 x 100 mm, I.D. 11 mm. Place in the cold (2-4°C) for 24 hours.

The following is the final procedure adopted: 1. Decapitate the rat and drain the blood, or anesthetize with ether. 2. Wet the hair over the abdomen to keep stray hairs from getting into the peritoneal cavity later, cut away a skin flap from the abdomen and fold over the chest, and then open the peritoneal wall with a mid-ventral incision using an electric cautery knife. 3. Drop 3 ml of heparinized (50 μg/ml) Hanks' balanced salt solution over the intestinal surface and gently massage the outside of the abdominal cavity for about 1.5 minutes. 4. Hold the intestines aside and remove the liquid (about 2 ml) with a drawnout medicine dropper from the space on each side of the spine. 5. Remove the tube containing the sucrose density gradient from the cold room, where it had been set up 24 hours earlier, and allow it to reach room temperature. Layer the peritoneal cell suspension over the sucrose solution, and diffuse the interface by gentle stirring with a thin glass rod. 6. Spin in a horizontal centrifuge for a total of 5 minutes, gradually increasing the speed to a maximum of 110 x

g, and then gradually reducing to zero. Allow to come to rest without braking. (A Size 1, Type SB, International Centrifuge was used, and after the tubes were placed in the carrier the rheostat was set to division 9, the cover was closed, the tachometer was connected, and the rheostat was advanced 1 division every 5 seconds until 740 rpm, division 16, was attained. Then the rheostat was set back 1 division to maintain this speed for 3⅓ minutes, after which deceleration was begun at the rate of 1 division every 5 seconds to zero.) 7. Clamp the tube on a vertical stand. Four layers of cells will be apparent, a top red cell layer, under it a dense white cell layer, below this a diffuse white cell layer and farther down the mast cell layer. Remove the mast cell layer with a 4 inch, 20 gauge, stainless steel hypodermic needle attached to a 2 ml syringe. (Samples of the layers were also removed with a 10 μl constriction pipette. Removal is aided by clamping the syringe or pipette to a vertical rack and pinion stand for controlled lowering and raising of the needle or pipette.) 8. To each ml of mast cell suspension in sucrose solution, add about 3 ml of distilled water and centrifuge for 5 minutes at 1500 rpm (452 x g). Remove most of the supernatant fluid and replace with Hanks' balanced salt solution. 9. To observe the morphology, mix 10 μl of cell suspension with an equal volume of 0.05% toluidine blue in 0.85% NaCl and shake by tapping the tube. Cell counts may also be performed on this preparation, although the unstained mast cells can be distinguished for counting.

Results. An example of results that can be obtained is given in Table I. The mast cell layer is distinct and separated sufficiently from the nearest white cell layer to permit easy removal. The isolated cells are uncontaminated by other cell types.

The positions of the cell layers after 5 minutes of centrifugation at 110 x g and room temperature are not the equilibrium positions that would be attained ultimately by longer centrifugation. However, the relatively large size and density of the mast cells combine to give them a sufficiently greater sedimentation rate than the other cells and this results in an adequate separation for isolation purposes. No advantage was gained when the centrifugation was prolonged to 10 or 15 minutes, or the centrifugal force was increased to 330 x g. In the latter instances the red cell layer moves part way through the white layers as the equilibrium positions are approached, and the bottom of the white cell layer gets closer to the mast cell layer.

TABLE I. Separation and Purity of Mast Cell Fraction from Peritoneal Washings of the Rat Using a Sucrose Density Gradient. (5 min. centrifugation at 110 × g and 25°C.)

Sample	mm below interface	Mast cells per μl	% mast cells
Original suspension		840	11.2
Red layer	1– 2	0	0
Heavy white layer	2– 5		
Diffuse " "	5– 9	0	0
Mast cell layer	13–15	920	100
Bottom of tube	30		
Final suspension*		1340	100

* Mast cell layer withdrawn, diluted, centrifuged and resuspended in 0.5 ml balanced salt solution.

Summary. The procedure of Padawer and Gordon for the isolation of mast cells from peritoneal washings was modified to employ a sucrose density gradient, and along with other changes in certain details, a procedure was elaborated that regularly provides mast cells 100% pure with respect to cell type in yields up to 87% with respect to the original washings.

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Forced Ovulation in Gonadotrophin-Treated Fasting Hens. (22477)

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(Introduced by R. M. Fraps.)
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In our earlier experiments(1-3) administration of gonadotrophic hormone (GTH) prevented the decrease of serum vitellin level and the occurrence of follicular atresia in the fasting hen. Follicles treated so were morphologically normal except for their longer stalks (2, Fig. 3). However, it has not been cleared whether these follicles show normal reaction to the ovulatory effect of LH or not. In previous work on ovulation in laying hens(3-7), the intravenous administration of luteinizing hormone (LH) induced premature ovulation of normally maturing follicles. Either first (C_1) or subsequent (C_s) follicles of regularly recurrent clutch sequences were ovulated within about 8 hours following LH injection (7,8). At comparable hours before expected ovulation, the sensitivity of C_1 follicles to the effect of LH was as much as 20 times greater than that of C_s follicles(8,9). However, at equal times from a preceding ovulation in the closed cycle, the reactivity of C_1 and C_s follicles was of the same order(10).

The present experiments were undertaken to elucidate the following 4 items on ovulation in the gonadotrophin-treated fasting hen: 1) Possibility of forced ovulation by LH administration; 2) Minimum dose of LH for the induction of ovulation; 3) Time interval between hormone administration and rupture of follicles; and 4) Possibility of forced ovulation of the immature small follicles (less than 2 cm diameter) by larger doses of LH injection.

Materials and methods. White Leghorn hens used in these experiments were put under fasting conditions similar to those described in a previous paper(1). For the purposes of items 1-3, birds expected from their clutch sequence to lay no eggs on the first 2 days of starvation were treated with follicle stimulating hormone (FSH) prepared by the Armour Laboratories (Lot No. PF-593-12X). Other groups were treated with gonadotrophin from pregnant mares' serum, PMS (Gonadogen, Upjohn, AYE DH 062AH) or LH (Armour, Lot No. 317-115), regardless of clutch sequences. Intramuscular injections were given for a period of 7-8 days. For forced ovulation, 0.5 mg, 0.1 mg and 0.05 mg of the LH preparation were injected into wing veins on the last day of starvation. Details pertaining to item 4 are noted below. The fluctuation of serum vitellin levels of the starved hens treated with GTH was determined by the precipitin reaction(1). Ovulation was first examined by cloacal palpation to determine the presence of an egg in the uterus and finally by postmortem inspection of the ovary for the presence of recently ruptured follicles.

Results. Table I shows the results of forced ovulation by intravenous injection of LH in GTH-treated fasting hens. Untreated fasting hens, as well as those injected with GTH intramuscularly(1-3), seldom lay eggs on the third day of starvation. This means that ovulation rarely occurs on the second day either of fasting or of GTH treatment.

In this experiment, however, on the 7th or 8th day of starvation, all of these treated hens except 3 were induced to ovulate 8 hours after injection of 0.5 mg or of 0.1 mg of LH-preparation, one of them ovulating two follicles. The injection of 0.05 mg of LH was without effect.

Fig. 1 shows a yolk in magnum at 9 hours after LH administration.

There occurred, however, no ovulation in 3 hens. At autopsy one of them (No. 2-214), injected with 0.5 mg of LH, had a large atretic follicle on her gonad, and the other 2, injected with 0.1 mg of LH, carried ovulable follicles similar to those in the birds responding by ovulation.

Thus it can be said that large follicles in GTH-treated fasting hens can be induced to ovulate by LH administration. The dose for

TABLE I. Forced Ovulation by Intravenous Injection of LH to Gonadotrophin-Treated Fasting Hens.

GTH pretreatment: Hormone	Daily dose (mg)	Days	Hen No.	LH inj. (mg)	No. of ovulated follicles	Inj. to ovulation (hr)	Vitellin level (prec. titer)	Ovary wt (g
FSH	.50	7	2–540	0.5	1	8	640	77.0
"	.25	8	2–526	"	1	7–8	320	38.5
LH	1.00	7	2–214	"	0	—	"	117.4
"	.50	"	3–654	"	1	8	"	89.2
"	.50	"	2–125	"	2	8	"	75.[illegible]
PMS	.50	"	2–381	"	1	8	640	54.3
FSH	.25	"	2–530	0.1	1	8	320	33.5
LH	.10	"	004	"	1	8	"	47.8
"	.10	"	002	"	0	—	"	26.[illegible]
PMS	.50	"	2–370	"	0	—	640	41.5
PMS	.50	"	2–377	0.05	0	—	640	42.1

TABLE II. Lack of Effect of Intravenously Injected LH on Immature Ovarian Follicles of Gonadotrophin-Treated Fasting Hens. No ovulated follicles in any of 5 hens.

GTH pretreatment: Hormone	Daily dose (mg)	Days	LH inj. (mg)	Vitellin level (prec. titer)	Ovary wt (g
FSH	.50	9	2.0	640	70.5
"	.25	"	2.0	320	33.5
"	.50	"	1.0	640	41.5
"	"	"	.5	640	32.7
"	"	"	.5	320	58.0

forced ovulation in 83% of injected hens is 0.5 mg hen, while the minimum dose is of the order of 0.1 mg.

The last experiment was to clarify item 4. All the hens recorded in Table II ovulated on the first 2 days of starvation (in contrast with the FSH treated hens of Table I), but failed to ovulate in response to LH injected even at the 2 mg level on the 9th day of starvation. At autopsy, the ovaries of these hens were found to carry numerous follicles of about 2 cm diameter (Fig. 2) but none of mature size. The effect of FSH in these hens appeared to be similar to the "over-all" action of pituitary gonadotrophin described by Phillips (11) rather than to the action of PMS.

As shown in these results, there occurred no forced ovulation of small immature follicles (about 2 cm in diameter) in the FSH-treated fasting hens by 2 mg of LH.

Summary. 1. Ovulation is induced by intravenous injection of LH preparation in

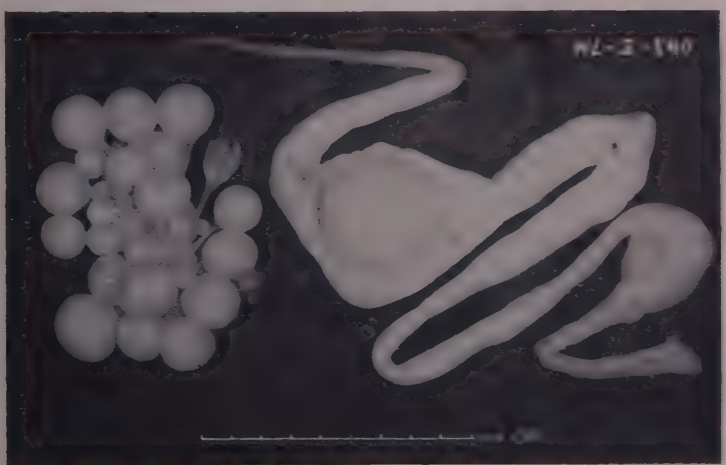

FIG. 1. Ovary and oviduct from FSH-treated fasting hen No. 2–540 (Table I), sacrificed 9 hr following inj. of 0.5 mg LH.

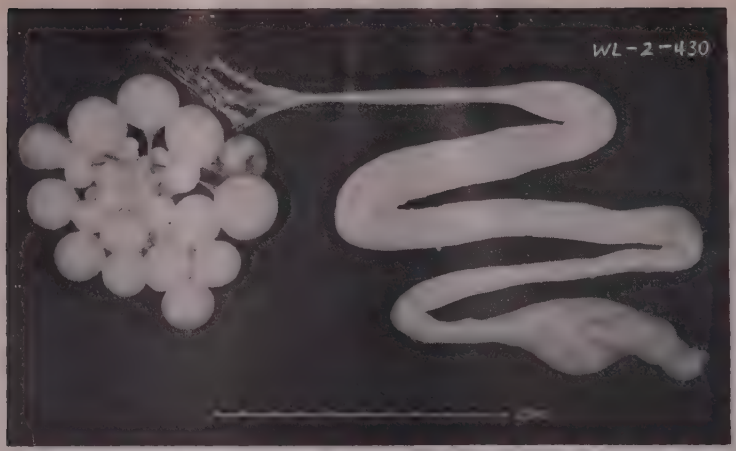

FIG. 2. Ovary and oviduct from FSH-treated fasting hen No. 2–430 (Table II), sacrificed 12 hr following inj. of 2.0 mg LH. Note absence of full-sized follicles.

gonadotrophin-treated 7-day starved hens. 2. 0.1 mg of the LH preparation used in this experiment is asumed to be the minimum dose for forced ovulation. 3. About 8 hours is the time interval between LH administration and rupture of follicles in such treated hens. 4. It was not possible to induce ovulation of small immature follicles by 2 mg of LH.

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Anticortisol Action of 2-Methyl-9(α)-Fluorocortisol.* (22478)

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It has been shown recently that 2-methyl-9(α)-fluorocortisol acetate (Me-F-COLA) possesses extraordinarily intense mineralocorticoid but comparatively slight glucocorticoid activity(1). This raised the question whether this new steroid, like other potent mineralocorticoids, can also block the antiphlogistic, thymolytic, splenolytic and catabolic actions of a glucocorticoid, such as cortisol. It will be recalled that aldosterone, the most active known naturally occurring mineralocorticoid, does possess such anticortisol actions(2). To verify this point, an experiment was performed with Me-F-COL, under essentially the same experimental circumstances under which aldosterone proved to inhibit cortisol.

Materials and methods. Forty female

* This work was subsidized by the Medical Research Board, Office of the Surgeon General, Department of Army.

TABLE I. Anticortisol Action of 2-methyl-9 (α)-fluorocortisol.

Treatment	Final body wt (g)	Wt gain (%)	Exudate (ml)	Thymus (mg)	Spleen (mg)
COLA	145 ± 3.1	−9	10.4 ± 1.3	114 ± 12.7	454 ± 36.4
COLA + Me-F-COL*	163 ± 1.9	+2	19.5 ± 1.4	158 ± 20.1	607 ± 21.7
COLA + Me-F-COL†	150 ± 2.5	−6	11.2 ± 1.9	61 ± 8.6	416 ± 33.7
COLA + DOCA	169 ± 1.9	+6	22.8 ± 1.8	233 ± 18.3	654 ± 31.8

* 10 μg. † 100 μg

Sprague-Dawley rats, with an average initial body-weight of 160 g (range: 154-166 g) were bilaterally adrenalectomized and subdivided into 4 groups, as indicated in Table I. Throughout the observation period these rats were maintained exclusively on "Purina Fox Chow" and tap water, without special salt supplements. Hormone treatment was initiated immediately after adrenalectomy. Cortisol acetate (COLA†) was given at the dose of 400 μg/day (Groups I-IV), Me-F-COL† at the dose of 10 μg (Group II) and 100 μg (Group III), and desoxycorticosterone acetate (DOCA†) at the dose of 100 μg/day (Group IV). The latter, a known active anticortisol compound, served merely as a control. All steroids were administered as microcrystals, subcutaneously, the daily dose being contained in 0.2 ml of aqueous suspension medium.

For the quantitative assessment of inflammation "granuloma pouches"(3) were prepared 48 hours after initiation of the hormone treatment, by the injection of 25 ml of air under the dorsal skin; this was immediately followed by the injection of 1.0 ml of 1% croton oil (in corn oil) into the air-space so created. All animals were killed on the 13th day after adrenalectomy. At this time the exudate was measured in milliliters, by aspiration into a graduated syringe, while the thymus and spleen were weighed after fixation in Susa solution.

Results. It is evident from Table I that, under these conditions, 10 μg of Me-F-COL was almost as effective as 100 μg of DOCA, in significantly inhibiting the loss of body-weight, the suppression of exudate formation and the involution of the spleen, which occur under the influence of COLA alone. Thymus involution was also inhibited, though not significantly. On the other hand, 100 μg/day of Me-F-COL proved to be virtually ineffective in all these respects.

Discussion. At first sight, it may appear to be paradoxical that Me-F-COL possesses a clear-cut anticortisol effect at low, but not at high, dose-levels. However, this is quite in agreement with what has been termed "the law of intersecting dose-effect curves." It had been found that, when a solution containing fixed proportions of COLA and DOCA is administered to adrenalectomized rats, the cortisol action (catabolism, inhibition of exudation, thymolysis and splenic atrophy) predominates at high and the opposite, desoxycorticosterone-type of activity, predominates at low dose-levels. This was ascribed to the fact that the DOCA activity rises rapidly to its optimum level, but then a "ceiling" is reached and raising the dose further will not increase the effect. The cortisol-type of activity, on the other hand, rises more slowly, but does not flatten out until it far exceeds the "ceiling" of its antagonist(4). Since Me-F-COLA is known(1) to possess considerable glucocorticoid activity (about 39 times that of cortisol), in addition to its much more evident mineralocorticoid action (about 90 times that of DOCA), it was to be expected that small doses of the compound would be prophlogistic, anticatabolic and stimulating to thymic and splenic development, while large doses would act inversely. In fact, it is conceivable that even smaller doses of Me-F-COL would have to be administered in order to bring out the maximum anticortisol action of this compound. In any event, our observations furnish additional evidence of some

† The authors gratefully acknowledge generous supplies of "Cortril" (COLA) from the Pfizer Laboratories, of 2-methyl-9(α)-fluorocortisol from Upjohn Co., and of "Cortate" (DOCA) from Schering Corp.

close relationship between mineralocorticoid and prophlogistic (anticortisol) potency. They demonstrate furthermore, by comparison with our earlier findings concerning aldosterone(2), that Me-F-COL—a synthetic steroid not known to occur in nature—at least equals, and probably exceeds, the anticortisol activity of the most potent natural mineralocorticoid.

Summary. In adrenalectomized rats bearing a "granuloma pouch," it has been demonstrated that 10 μg/day of 2-methyl-9(α)-fluorocortisol (Me-F-COL) definitely antagonizes the catabolic, antiphlogistic, thymolytic and splenic atrophy-producing actions of 400 μg/day of cortisol acetate (COLA). Higher doses of Me-F-COL are less effective in these respects. Since Me-F-COL possesses some glucocorticoid potency, in addition to its strong mineralocorticoid effect, the comparatively low anticortisol activity of this compound, when it is given at high dose-levels, is in agreement with expectations based on the "law of intersecting dose-effect curves."

Addendum. Since this manuscript went to press we were able to show that Me-F-COL is more than 100 times as active as DOCA in producing nephrosclerosis and cardiovascular hyalinosis in suitably conditioned rats (Selye, H., and Bois, P., *Endocrinology*, in press).

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Diffusion Coefficient of Urate for Human Connective Tissue Membrane. (22479)

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Determinations of the diffusion coefficient of urate for connective tissue have not as yet been reported. The present study was carried out on 10 samples of the cerebellar tentorium, obtained fresh at autopsy, using the diffusion method of Johnsen and Kirk(1). In this procedure the diffusion rate of a compound from one fluid phase to another through a membrane is measured under sterile conditions and at a constant total pressure.

Methods. The experiments were conducted at 37°C. A solution of sodium urate (190-197 mg %) in Krebs' phosphate buffer, pH 7.4, was used in the donor compartment of the apparatus, and phosphate buffer in the recipient compartment. The urate solution was prepared fresh daily by addition of 20 ml 0.1 N NaOH solution to 200 mg of uric acid contained in a beaker. The beaker was placed in a water bath at 60°C, and the solution stirred vigorously. 60 ml of Krebs' phosphate buffer, preheated to 60°C, were then added; after adjustment to pH 7.4 the solution was made up to 100 ml with phosphate buffer, and, after cooling to 25°C, was filtered through a Whatman No. 2 filter paper. The purpose of the cooling and subsequent filtration was to insure against precipitation of urate from the solution during the experiment. For the diffusion experiment the tentorium sample was rinsed with sterile buffer solution and a portion of the membrane inserted in the diffusion apparatus. Both the donor and recipient solutions were heated to 37°C before they were introduced into the apparatus. 45 minutes were allowed to elapse for establishment of temperature equilibrium and for initial penetration of urate through the membrane before the first samples were withdrawn for analysis. In each experiment 2 diffusion periods of 90

minutes were used. The sample withdrawals were carried out in immediate succession from the donor and recipient compartments at the beginning and end of a diffusion period; this made it possible to employ the equation given by Pletscher *et al.*(2) for calculation of the diffusion coefficient. The diffusion coefficient is defined according to Hill(3) as the number of units of the compound diffusing through a 1 cm^2 area of the membrane in 1 minute at a concentration gradient of 1 unit per ml per cm.

The determination of the urate content of the samples was performed by enzymatic differential spectrophotometry(4), using a Beckman DU spectrophotometer. To prevent precipitation of urate through cooling the collection of the samples from the diffusion apparatus was made in test tubes heated in a water bath to 50°C. Before analysis the samples from the donor compartment were diluted with glycine buffer in the proportion of 1:250, whereas dilutions of 1:2.5, 1:10, and 1:25 were employed for the first, second, and third samples, respectively, from the recipient side. The dilutions were carried out in such a way that a final glycine concentration of 0.067 M was obtained. At the end of the experiment the part of the membrane which had been delimited by the circular openings of the metal diaphragms of the apparatus was carefully cut out and its area and weight were determined. From these values the mean thickness of the membrane was calculated.

Results. The results of the diffusion coefficient determinations are presented in Table I. The average urate diffusion coefficient value found was 0.000155 ± 0.000011.

TABLE I. Diffusion Coefficient of Urate for Human Cerebellar Tentorium.

Sex	Age (yr)	Thickness of tentorium (mm)	Diffusion coefficient of urate 1st period	2nd period	Avg
♂	24	.518	.000111	.000119	.000115
♀	24	.568	.000131	.000116	.000123
♂	31	.483	.000157	.000150	.000154
♂	37	.517	.000183	.000197	.000190
♀	50	.480	.000149	.000138	.000145
♂	55	.521	.000168	.000174	.000171
♂	55	.310	.000124	.000133	.000129
♂	56	.683	.000210	.000256	.000233
♀	57	.697	.000176	.000140	.000158
♂	67	.648	.000137	.000124	.000131
		Mean	.000155	.000155	.000155

No significant correlation was noted between the age of the individuals from whom the connective tissue membranes were derived and the coefficient values measured.

Summary. Determinations were made at 37°C by the method of Johnsen and Kirk of the diffusion coefficient of urate for a human connective tissue membrane (cerebellar tentorium). The urate analyses were performed by enzymatic differential spectrophotometry. The average coefficient value found in experiments on 10 samples was 0.000155.

The authors wish to thank Dr. J. J. Connor for assistance in obtaining tissue specimens.

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Further Studies on Lathyrism in the Rat.* (22480)

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It was reported(1) that β-aminopropionitrile and aminoacetonitrile fed to weanling male rats produced extensive skeletal lesions similar to those found in rats fed *Lathyrus odoratus* seeds(2). Bis(β-cyanoethyl)amine produced minimal skeletal lesions when fed for an extended period; the behavior of rats on this compound was suggestive of cerebellar lesions as reported by Bachhuber *et al.*(3). β-Aminopropionitrile and aminoacetonitrile, when injected subcutaneously, are at least as active in the production of skeletal lesions in rats as when given orally. Daily injections of .02 g and .01 g of β-aminopropionitrile and .003 g and .005 g of aminoacetonitrile hydrosulfate to weanling rats fed the standard laboratory pellet diet produced very severe skeletal lesions in 15 to 20 days. β-aminopropionitrile injected in daily doses of .005 g and .001 g produced only slight skeletal lesions in 27 days. Three rats fed diets‡ containing 0.1% methyleneaminoacetonitrile developed very severe skeletal lesions within a week. These animals died with severe inanition from 6-11 days after the onset of the diet. The roentgenograms demonstrated extremely abundant periosteal new bone formations in the femurs and humeri and extensive clefts in the widened epiphyseal plates of the long bones were observed in the histological sections. Another group of 3 weanling rats was fed a diet containing 0.05% of methyleneaminoacetonitrile for 64 days. These rats developed extensive degenerative arthritis, periosteal new bone formations in the femurs, and moderate widening and disarrangement of the epiphyseal plates. A diet containing 0.01% of the same compound was fed to another group of 3 weanling rats for 64 days. The roentgenological and histological examinations of the skeleton of these rats did not reveal any lesions. It is possible that after ingestion the methyleneaminoacetonitrile hydrolyzes into aminoacetonitrile and formaldehyde. However, this appears unlikely because the study of the severity of the skeletal lesions in relation with the daily intakes of these two nitriles indicates that the methyleneaminoacetonitrile is probably more active than the aminoacetonitrile. The report that β-mercaptoethylamine(4) produces the skeletal lesions of lathyrism could not be verified. Six weanling rats placed on a diet§ containing 0.3% of this compound for 35 to 64 days did not develop any skeletal lesions detectable in the roentgenograms or in the histological sections. In a previous paper(1) a number of nitriles were reported which did not produce lathyrism when fed to rats. Similarly, no lesions have been observed after feeding to weanling rats the following diets§ for periods of 5 to 10 weeks: 0.5% succinonitrile, 0.5% hydroacrylonitrile, 0.2% acrylamide, 0.1% acrylamide, 0.1% cyanamide, 1% α-cyanoacetonitrile, and 1% cyanoguanidine. It has been reported that diets high in vit. E(5), in casein(6), and in gelatin(7) give partial protection from the lesions of lathyrism. We have not been able to corroborate these reports. A group of 20 four-week-old rats fed a diet containing 50% *Lathyrus odoratus* seeds‖ were given alpha-tocopherol by mouth in doses of 120-300 mg per week for a period of 13 weeks. Another group of 20 rats of the same age and fed the same diet but without the supplementary feeding of alpha-toco-

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‡ The control diet consisted of casein, 18%; yeast, 10%; Crisco, 5%; Wesson Oil, 5%; sucrose, 29.6%; starch, 29.7%; salt mixture, 2.5%; haliver oil, .2%; choline, .04%.

§ Same diet as in footnote ‡.

‖ The 50% *Lathyrus odoratus* diet used in these experiments was as follows: casein, 10%; yeast, 10%; Crisco, 5%; Wesson Oil, 5%; sucrose, 8.6%; starch, 8.7%; salt mixture, 2.5%; haliver oil, 0.2%; *Lathyrus odoratus* pea, 50%; choline, 0.04%.

TABLE I. Antioxidants Incorporated into 50% *Lathyrus odoratus* Diet.

Compound	% in diet	Days on diet
2,6-Di-tert-butylphenol	1	35
Nordihydroguaiaretic acid	.5	63
N,N'-Diphenyl-p-phenylenediamine	.5	49
2,6-Di-tert-butyl-p-cresol	.5	49
Gum guaiac	.5	49
n-Propyl gallate	.5	49
Butylated hydroxyanisole	.5	49
4-tert-Butylpyrocatechol	.23	42
DL-β-3,4 dihydroxyphenylalanine	.28	42
Tyrosine ethyl ester	.29	42
Nordihydroguaiaretic acid + L-Methionine	.1 + .1	35
Nordihydroguaiaretic acid + Citric acid	.1 + .1	35
β-Conidendrol	1	23
α-Tocopherol	120-300 mg/wk	91

pherol served as controls. The incidence of dissecting aneurysm of the aorta and the severity of the skeletal lesions as judged from the roentgenograms and the histological examinations was similar in these two groups. In another experiment a group of 22 weanling male rats was fed a synthetic¶ vit. E deficient diet for a period of 4 to 7 months. All these rats developed severe testicular atrophy with degeneration of the germinal epithelium. The animals not sacrificed until the sixth or seventh month of this diet developed muscular dystrophy with paralysis and some died suddenly. Histological and roentgenographic studies of these animals revealed no lathyrism-like lesions.

To evaluate any possible curative or protective effects of other antioxidants on the development of lathyrism, the compounds listed in Table I were tested. Groups of 3 to 9 weanling rats were used for every compound. Roentgenograms of the rats under ether anesthesia were taken every 2 weeks and at the postmortem examination the femurs and upper tibiae were obtained for histological studies. Hematoxylin and eosin and Mallory stains were used. In no instance could a protective effect be demonstrated. Of interest, however, is the observation that the rats on these compounds often ate less than control rats. In these cases lesions in experimental animals were less severe than in controls. When the intake of control animals was limited to that of the experimental, no difference in severity of lesions could be demonstrated. To increase the intake of the *Lathyrus* pea diet plus the antioxidant, lower concentrations of nordihydroguaiaretic acid were used with the synergists citric acid and methionine. No protective effect could be demonstrated.

A high concentration of casein (27.3% of diet), when incorporated into 35% and 50% *Lathyrus odoratus* diet, showed no demonstrable protective effect on development of skeletal lesions. No rupture of the aorta occurred in the 7 weanling rats fed the high casein diet. However, a similar number of controls fed *Lathyrus* diet alone likewise did not show this lesion.

Other compounds tested for possible protective effects on lathyrism in the rat are listed in Table II. Rutin was tested because of its possible antifragility action; sodium thiosulfate, for its effectiveness in cyanide poisoning; γ-aminobutyronitrile, pantothenic acid, taurine, cystine, propylamine, ethylamine, propionaldehyde oxime, and acetaldehyde oxime as possible competitive inhibitors of aminonitriles; glucuronolactone, as a detoxifying agent for amines; sodium salicylate, because of its known clinical action in arthritis; glucosamine because of its importance in connective tissue structures. Groups of 3 to 9 rats were used for every compound

TABLE II. Compounds Incorporated into 50% *Lathyrus odoratus* Diet.

Compound	% in diet	Days on diet
Rutin	1	35
Sodium thiosulfate	.5	49
Casein	27.3	63
Cystine	2	63
γ-Aminobutyronitrile	4	42
d-Pantothenic acid	1	39
D-Glucosamine	1	39
Taurine	8.9	42
Glucuronolactone	10	41
Sodium salicylate	1	21
Propylamine*	1	24
Ethylamine*	2	24
Propionaldehyde oxime	2	12
Acetaldehyde oxime	2	10

* Fed as salt of HCl with $NaHCO_3$.

¶ The tocopherol deficient diet was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio.

TABLE III. Excretion of Sulfur in Rats Fed Control (Stock Rockland Pellet) Diet, *Lathyrus* Pea Diets, and *Lathyrus* Pea plus 0.5% Nordihydroguaiaretic Acid (NGA) Diet.

Diet	No. of rats	No. of days	Total S, mg/day/rat, mean	Total S, SO_4-S, mean
		Series I		
Control	2	10	11.25	57.1
50% pea	4	20	5.95	65.5
		Series II		
Control	2	4	5.31	76
35% pea	2	4	4.17	85
50% "	2	4	3.37	69
.5% NGA	3	4	4.06	43.5
		Series III (intake controlled)*		
Control	2	2	3.36	61.4
50% pea	3	2	3.10	53.5
.5% NGA	2	1	3.71	4.3

* In Series III, intake of all 3 groups was limited to that of group ingesting the least food. Series I and II were permitted unlimited intake.

incorporated into a 50% *Lathyrus odoratus* diet. Roentgenograms of the animals under ether anesthesia were obtained every 2 weeks and at postmortem examination the femurs and upper tibiae were obtained for histological studies. No protective action could be demonstrated with any of these compounds.

The urinary excretion of sulfur in rats fed a *Lathyrus* pea diet and the same diet plus 0.5% of nordihydroguaiaretic acid was studied together with the sulfur excretion in control rats fed standard laboratory pellet diet (Rockland) (Table III). Total sulfur excretion by 3 series of control rats varied with their intake; 57 to 76% of the total sulfur was excreted as sulfate. The rats fed *Lathyrus* pea diets excreted 53 to 85% of the sulfur as sulfate. When nordihydroguaiaretic acid diet was fed to rats for one day (series III) only 4% of the excreted sulfur was in the oxidized form, compared to 53.5 and 61.4% in rats on pea and control diets restricted to the same intake of food as rats fed the antioxidant. However, rats fed nordihydroguaiaretic acid for 4 days showed considerable recovery of their ability to oxidize sulfur, excreting 43.5% of the total as sulfate. Partition of urinary sulfur by rats fed *Lathyrus* peas, in comparison with control rats, gave no evidence of altered sulfur metabolism in lathyrism.

The deposition of sulfur-35 in the epiphyseal plates, bones, and aorta of rats with Lathyrism was investigated by radioautography. Five 7-week-old male rats were used. Three rats received a 50% *Lathyrus odoratus* diet since 22 days of age. Two rats received the standard laboratory pellet diet and served as controls. Approximately 60 μc of carrier-free sulfur-35 in the form of sodium sulfate in 0.3 ml distilled water was injected intraperitoneally into each animal. They were sacrificed with chloroform 20 hours later. One rat on the pea diet was found dead with a hemothorax due to ruptured dissecting aneurysm of the aorta. The knees including lower femur and upper tibia, shoulders, hip joints, lower thoracic and upper lumbar vertebrae, and thoracic aorta were removed from each animal. Preservation was in neutral formalin and decalcification was done with versene. Sections were mounted on autograph emulsion(8) with exposures of about one week. The S^{35} is chiefly in the form of chondroitin sulfate(9).

Radioautographs demonstrated deposition of S^{35} in the epiphyseal plates in about equal amounts in experimental as in control animals. A high concentration of S^{35} was observed in experimental rats at the site of periosteal detachments overlying areas of new bone formations. This, of course, was not seen in control rats. S^{35} was deposited in small amounts in the thoracic aorta of both control and experimental animals. No greater concentration of S^{35} was observed in the aorta with the dissecting aneurysm. The uptake of S^{35} by chondroitin sulfate was the same in experimental as in control animals and, therefore, it may be assumed that the sulfur content of the chondroitin sulfate is not greatly altered in lathyrism. These findings appear to be in accordance with the work of Bauer *et al.*(10).

Summary. 1. Male rats injected subcutaneously with β-aminopropionitrile and aminoacetonitrile developed the same skeletal lesions of lathyrism as did those fed these compounds. 2. Methyleneaminoacetonitrile fed to rats produced very severe skeletal lesions and is probably more active than the

aminoacetonitrile. 3. Diets high in casein, gelatin, vit. E, and other antioxidants did not protect the rats fed *Lathyrus odoratus* seeds from developing skeletal lesions. 4. Radioautographs of the skeleton after injection of S^{35} and studies on sulfur excretion of rats fed *Lathyrus odoratus* peas and rats fed a control diet showed that the metabolism of sulfur is probably not altered in lathyrism.

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Some Clinical Conditions Affecting Urinary Excretion of Non-Dialyzable Hexosamine.* (22481)

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In a preceding communication(1), the normal levels of adult human urinary excretion of non-dialyzable conjugated hexosamine were reported to be 32.4 ± 7.2 mg/day for males and 25.9 ± 5.8 mg/day for females. The hexosamine appeared to be principally glucosamine. Other recent publications(*e.g.,* 2-5) related to this subject indicate or suggest that more than one conjugate contributes to the total hexosamine present in the non-dialyzable fraction of both normal and pathological urine, part being present as a result of glomerular filtration and part originating from the urinary epithelium.

This communication reports the results of investigations of "bound" hexosamine excretion in certain clinical conditions. These determinations were undertaken in an effort to distinguish some aberrations affecting bound hexosamine excretion, which in turn might further elucidate its origin and nature.

Methods. The procedure was the same as that previously described(1). Twenty-four-hour urine specimens were accurately collected and preserved with phenylmercuric nitrate. As a check on the entire procedure, samples of urine from healthy individuals were included at intervals.

Results. The results are expressed as mg glucosamine per 24 hours. Initially, urines from patients with some selected conditions were examined. These preliminary experiments indicated an increase in bound hexosamine in several of these (Table I). Urolithiasis, atherosclerosis, and pregnancy were further studied.

Urolithiasis. Neither the chemical type of urolite, a low-calcium diet, nor the presence of infection (in each of these cases minimized by concurrent antibiotic or chemotherapy) appeared to influence the results. An elevated bound hexosamine excretion was found in all cases (Table II). This was particularly striking in cases with large or actively growing stones.

Three male patients were included who had

* We gratefuly acknowledge the courtesy of the following in supplying urine specimens and case histories: Drs. William H. Boyce and Emery C. Miller, Bowman Gray School of Medicine of Wake Forest College, Dr. Robert Quinn, Vanderbilt University Medical School.

TABLE I. Bound Hexosamine Excretion Found for Some Conditions.

Diagnosis	mg hexosamine/day
Males	
Idiopathic myocarditis	48.7
Pernicious anemia	53.8
Pulmonary emphysema	29.5
Total colectomy, ilio-proctostomy, ulcerative colitis (on cortisone)	19.1
Controlled diabetes mellitus	48.0
Bilateral cutaneous ureterostomy	13.0
Chronic osteomyelitis	31.9, 26.0
Bilateral nephrocalcinosis	48.7
Females	
Post-infection cystitis	45.7
Polycystic kidney disease	19.0, 24.0, 39.5, 29.2
Hyperthyroidism	47.7
Hypothyroidism	29.3
Adrenal hyperplasia (on cortisone)	32.0
Glomerulonephritis	13.3
Controlled diabetes mellitus	25.3
Subclinical diabetes insipidus	30.1
Rheumatoid arthritis (on ACTH)	32.1
" " (no medication)	30.3

normal urinary tracts at the time of the urine collection, but with a past history of formation of a 2 mm, or smaller, stone. These patients had subnormal levels of bound hexosamine: five samples averaged 22.7 mg per day.

Two days on a high (800 mg/day) calcium diet did not elevate the bound hexosamine level of a normal male, 28.1 mg being found.

Atherosclerosis. This group, comprised mostly of males (44-55 years old), had typical clinical evidence of fulminating atherosclerotic disease, as reflected symptomatically in electrocardiograms, and by serum lipoprotein and serum cholesterol levels. A significant increase in bound hexosamine excretion was invariably present (Table II).

Pregnancy. Specimens were obtained from 4 multiparas and 5 primaparas during pregnancy and in 2 subjects also subsequent to parturition. An augmented appearance of bound hexosamine regularly occurred during the last 5 months of pregnancy, with a return to the normal value in about 2 months (Table III).

Although 2 of the patients with renal calculi listed in Table II had previous unilateral nephrectomies, they still gave values no different from the others, suggesting that the urothelium of the collecting system is not a major contributor of bound hexosamine in this condition (but *cf.* ureterostomy patient, Table I).

Chemical studies. Since any determination of hexosamine in biological material is liable to error from many sources, comparisons have been made using other technics of hexosamine measurement. Parallel samples of dialyzed, hydrolyzed urine were assayed by the usual procedure(1), by Prodi's(6) modification of it, or by the method of Dische and Borenfreund(7). The method of Prodi (in 10 normal and clinical samples) was less precise, but gave values ranging within 20 mg per day of the present method, while that of Dische and Borenfreund gave much higher values, having no apparent constant relationship to those obtained by our technic. The latter results probably reflect an interference(8) by the hexoses present(4).

Hexosamine was estimated by the Prodi technic, along with samples including 1 ml of 3.2% $Na_2B_4O_7 \cdot 10H_2O$, according to Tracey (9). The volatile fraction from the Elson and Morgan reaction is affected in the same way by borate as is the total reaction product, *i.e.*, glucosamine had its color intensity reduced about 75% by borate treatment while that of galactosamine was reduced only about 50%. Samples of dialyzed, hydrolyzed normal urine so treated exhibited an average

TABLE II. Bound, Non-Dialyzable Hexosamine Excretion by Normal Individuals, and Patients with Atherosclerosis or Urolithiasis.

Disease	No. subjects ♂	No. subjects ♀	No. specimens ♂	No. specimens ♀	Hexosamine/day (mg avg ± S.D.) ♂	Hexosamine/day (mg avg ± S.D.) ♀	Probability of significance ♂	Probability of significance ♀
None*	14	13	30	18	32.4 ± 7.2	25.9 ± 5.8		
Urolithiasis	15	16	20	27	50.0 ± 11.9	53.8 ± 22.5	$P < .001$	$P < .001$
Atherosclerosis	7	1	16	1	46.4 ± 9.4	56.0	"	

* From reference (1).

TABLE III. Prenatal and Postpartum Values for Non-Dialyzable Hexosamine for 9 Subjects.

Weeks pregnant	8	12	16	20	24	28	32	36
mg hexosamine	25.0		26.9					
	32.3	19.0	26.5	39.9	43.9	47.3	43.0	36.0
	29.7		25.0	56.8	51.4	52.2	54.6	36.8
	25.2		28.6	51.0		52.8	56.5	62.5
							192.5*	
Weeks post-part.	1	2	8	9				
mg hexosamine	107.8	43.9	28.9	49.6				
			28.4	25.3				
				31.6				

* Proteinuric.

color reduction of 73% after borate treatment, confirming both the specificity of the Prodi procedure and the predominance of glucosamine over galactosamine.

Discussion. The finding of an increased hexosamine excretion by patients with urinary calculi is in accord with Boyce *et al.*(2), and others(10,11), who find a similar increase in hexosamine-containing biocolloids in the urine and urinary tracts of such patients.

The increased mucinous secretion by the epithelium of the urinary tract in pregnancy is accompanied by an increase in urinary mucoprotein(12), which is evidently reflected in bound hexosamine measurement. Since these specimens were not obtained by catheterization, the vaginal epithelium could also have contributed. The rise in the hexosamine-containing plasma proteins during pregnancy (13) could be connected with these observations.

Summary. 1. The quantity of non-dialyzable hexosamine in human urine increases significantly and consistently during the last half of pregnancy, and in patients with renal calculi or atherosclerosis. 2. The results indicate that this hexosamine may have a variety of origins.

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Adenosine as Growth Factor for Chicks Fed Purified Diet.* 22482

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Barnett and Bird(1) found that chicks responded consistently to sources of unknown growth factors if fed a purified basal diet purposely contaminated with feces of older chickens.

A concentrate prepared from penicillin fermentation residue was furnished by E. R. Squibb and Sons, and stated to be highly active by chick assay and by microbiological assay. Upon fractionation it yielded, at one stage, a considerable quantity of a crystalline material. Ultimate analyses and comparative infrared spectra revealed that this material was adenosine(2). Adenosine proved to be inactive in the microbiological assay, but active in the chick assay.

Methods. The microbiological assay procedure followed was that of Schaefer and McCormack(3). Day old New Hampshire X Silver Cornish chicks from depleted dams(1) reared in electrically heated batteries with raised wire floors were used in all chick assays. Ten chicks were started in each experimental lot. The purified ration in Table I, and the method of fecal contamination(1) were used except in Exp. 2 where the feces were omitted. All supplements were added at the expense of sucrose. During Exp. 2, a deficiency of vit D developed, and the level of this ingredient was increased from 600 ICU to 1200 ICU per kg of ration. Even at this level mild symptoms of rickets appeared and the practice of administering 2 drops of haliver oil twice weekly was followed subsequently. This eliminated the vit. D deficiency. Some cases of ataxia and some deaths were observed in groups not receiving fish solubles. Inasmuch as maximum responses were obtained, in many cases, only when 2 widely differing sources of unidentified growth factors were included in the diet, the initial test of adenosine was made with dried whey or fish solubles. Subsequent tests have always included one group in which adenosine was the only supplement. The adenosine was a commercial sample prepared from yeast, and stated by the supplier† to be chromatographically pure. The levels of adenosine were chosen arbitrarily, but were known to be considerably higher than the level furnished by the abovementioned concentrate. Adenosine was fed at 150 mg kg of diet in the first 2 experiments and at 300 mg kg in the last 3 trials.

Results. In the first experiment adenosine elicited a pronounced growth response when added to the diet which contained 2% dried whey. In the second experiment there is some question about the significance of response to adenosine in the presence of dried whey because only one male and 4 females remained in the control group at 4 weeks of age. In subsequent experiments no response to adenosine occurred in the presence of whey. Adenosine gave a definite response in only one of the 5 experiments when fed in addition to 3% of fish solubles. In 2 other experiments there was a suggestion of a response in the presence of fish solubles (Table II).

In all 4 of the experiments in which adenosine was fed alone a response was obtained; however, in Exp. 3 the response occurred only in the males. Table III shows the average of all males from this series of experiments which received the basal diet and the average of all males which received only adenosine supplementation. The same data are presented for the females and the average of the male and female averages. A statistical analysis by the method of Titus and Hammond(4) showed that the males which received adenosine were significantly larger than the males which received the basal diet. Also the unweighted mean of the sexes in the adenosine group was significantly greater

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† Nutritional Biochemicals Corp.

TABLE I. Basal Ration Used in Chick Assay for Unidentified Growth Factors.

	g/kg
Sucrose	628
Isolated soybean protein (Drackett C-1)	250
*Salts premix	60
Soybean oil	45
DL Methionine	6
Glycine	3
Choline chloride	2
Inositol	1
†Vit premix in sucrose	5
	1000

Plus the following (per kg):

Alpha tocopherol acetate	10 mg
Vit D	600 ICU
" A	15,000 USP

* Salts premix		† Vit premix	
$CaCO_3$	3803.2	Vit B_{12}	.02 mg
K_2HPO_4	2580	Biotin	.2
$CaHPO_4 \cdot 2H_2O$	1190	Menadione	.5
NaCl	1340	Pyridoxine	4
$MgSO_4 \cdot 7H_2O$	816	Pteroylglutamic acid	4
Fe Citrate $6H_2O$	220	Riboflavin	6
$MnSO_4 \cdot H_2O$	40	Ca Pantothenate	20
KI	6.4	Thiamin	10
$CuSO_4 \cdot 5H_2O$	2.4	Niacin	50
$ZnCl_2$	2.0	p-Aminobenzoic acid	100
	10,000.0	Sucrose to total	5 g

than in the basal group. However, the females which received adenosine were not significantly larger than the controls.

In each of the 4 experiments in which a comparison is possible, 3% of fish solubles gave a more pronounced growth response than adenosine. In 2 of 4 experiments whey improved growth in the presence of fish solubles.

TABLE III. Effect of Adenosine Supplementation—Exp. 2-5. Mean chick wt at 4 wk in g.

Treatment	♂	♀	Avg of ♂ & ♀ means
Basal	176 (22)	173 (18)	174 (40)
Adenosine*	222 (11)	187 (13)	204 (24
Difference	46†	14‡	30†

* Fed at 150 or 300 mg/kg of diet.
† Significant at 5% level(4).
‡ Not significant.
Figures in parentheses represent No. of birds.

Discussion. The response to adenosine, the greater response to fish solubles, and the fact that in most cases adenosine failed to improve the diet containing fish solubles suggest that adenosine might be one component of the growth factor complex of fish solubles. The existence of such a complex is indicated by the reports of Morrison *et al.*(5) and Couch *et al.* (6) that the ash accounts for part, but not all of the growth promoting effect of fish solubles. Other compounds similar to adenosine including adenine, kinetin(7), 5′-adenylic acid, and adenosine triphosphate have been studied for their growth promoting effects. Preliminary results indicate that none is superior to adenosine.

In these experiments and in many not reported here maximum responses were always obtained with the combination of fish solubles and whey. In some experiments whey did not give an additional response when added to the diet containing fish solubles. In some previous experiments the converse was true, *i.e.*,

TABLE II. Response from Adenosine Supplementation. Wt in g at 4 weeks.*

Additions to basal diet	Exp. 1		Exp. 2		Exp. 3				Exp. 4		Exp. 5	
	Adenosine level, mg/kg of diet											
	0	150	0	150	0	300	0	300	0	300	0	300
							♂ only					
None	152	—	192	224	169	167	175	197	152	196	180 216‡	215 293‡
2% dried whey	153	192	163†	190	178	158	165	171	149†	147	191 278‡	278‡†
3% fish solubles	244	227	253	271	224	268	253	303	251	270	239 311‡	296‡
2% dried whey + 3% fish solubles	242		296	274	280		309		244			

* All figures are avg of male and female averages except column designated "males only."
† Only 1 remaining male or female with 2 to 5 of opposite sex in these groups.
‡ Protein (Drackett C-1) increased to 40%.

fish solubles sometimes failed to give an additional response in diets containing whey. One supplement alone sometimes failed to elicit a response, as in the experiments reported here.

These results are not interpreted as contradicting reports from other laboratories that fish solubles and whey contain different unknown factors. They seem to indicate that the dietary requirements for the different unknowns vary from time to time for obscure reasons.

Other species have been observed previously to respond favorably to dietary purines and purine derivatives under certain conditions. Frost and Elvehjem(8) noted a growth response when adenine nucleotides were fed to rats on a low niacin diet. Huff and Bosshardt(9) found that mice which were fed 2% succinylsulfathiazole in a low fat diet gave a growth response to purines and purine derivatives.

Summary. In 5 experiments growth of chicks was increased by feeding adenosine at 150 or 300 mg/kg of purified diet. Fish solubles at the rate of 3% of the diet gave a more pronounced response than did adenosine. Adenosine did not consistently elicit a response in the presence of either fish solubles or dried whey.

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Influence of Season on EEE Infection in English Sparrows. (22483)

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Clinical eastern equine encephalomyelitis (EEE) is not observed in man or horses in the United States during the winter months. Antibody surveys and attempts to isolate virus from wild birds and mosquitoes indicate that the virus is not active or is at a very low level of activity during the winter in the southern United States(1). Cessation of mosquito activity may explain this pattern in more northern areas but some species are active in considerable numbers throughout the winter in lower southern areas. It has been shown that lower temperatures such as occur in southern areas during the winter do not adversely influence the ability of mosquitoes to transmit the virus(2). Neither can the lack of virus activity be correlated with the absence of a susceptible bird population since susceptible birds migrate into the southern areas during the winter months. It has been shown that changes in day length and an internal reproductive rhythm influence the physiology, reproduction, and migratory behavior of birds(3,4). It is possible, therefore, that the cessation of virus activity may be due to changes in response to the virus in birds brought about by seasonal changes.

The present study was conducted to determine whether the response of birds to EEE infection was altered by seasonal influences.

Materials and methods. English sparrows (*Passer domesticus domesticus*) were trapped

near Montgomery, Alabama. The reproductive status of the sparrows was determined by bill color in the males(4), sexual display, and nesting activity. The breeding season in this locality extends from about February 15 to September 15. All captured birds were held from 3 days to a week in 2′ x 3′ x 3′ wire cages to allow adjustment to captivity before inoculation, with the exception of a few birds held for a month in a roofed 8′ x 8′ x 8′ wire cage. EEE virus used was strain AR167, which was isolated from *Culiseta melanura*(5) and had received 2 mouse intracerebral and one chick embryo passage. Birds were inoculated subcutaneously over the breast with 0.03 ml of varying virus dilutions. Amounts of virus inoculated are expressed as mouse intracerebral LD_{50} units of virus. Birds were bled from the jugular vein. At each bleeding 0.1 ml of blood was withdrawn and diluted immediately with 0.9 ml of 10% horse serum in buffered water. Virus titrations and neutralization tests were performed by the intracerebral inoculation of 3-week-old CFW mice. Tests for neutralizing antibody were made by mixing the 1:10 diluted blood samples with 10-fold dilutions of virus. LD_{50} endpoints were calculated by the method of Reed and Muench(6). When deaths occurred, birds were autopsied and suspensions of brain and liver were inoculated into mice for the isolation of virus.

TABLE I. EEE Infection in English Sparrows.

Date of inoc.	LD_{50} inoc.	Sex	Max blood virus titer	Duration of viremia (days)	Died or survived
11/19/53	.4	♂			S
		♂	5.8	4	S
		♀	3.3	1	D
		♀			S
	.13	♂			S
		♂			S
		♀			S
		♀	7.3	2	D
	.04	♂			S
		♂			S
		♀			S
		♀			S
2/ 2/54	1.0	♂	5.5	4	D
		♂	5.5	3	S
		♀	5.5	3	D
		♀	5.5	5	S
	.32	♂			S
		♂	6.5	3	D
		♀	7.0	3	D
		♀			S
	.1	♂			S
		♂			S
		♀			S
		♂			S
4/27/54	1.3	♀			S
		♀	6.5	4	D
		♂	7.5	2	D
		♂			S
	.4	♀			S
		♀			S
		♂	7.0	1	D
	.13	♀			S
		♀			S
		♂			S

Results. Eight groups of birds were inoculated between July 6, 1953 and April 27, 1954. Each group was divided into 3 subgroups; one of the subgroups received an inoculum expected to infect none of the birds; a second received an inoculum expected to infect half; and a third received an inoculum expected to infect all. The dates of inoculation, amounts of virus inoculated, maximum viremia titers, duration of viremia, and outcome in representative groups are shown in Table I.

The smallest dose of virus which infected any bird (3/10) was 0.13 LD_{50} unit. Doses of 1.3 LD_{50} or greater always infected at least half of the birds. Doses of 3.2 LD_{50} or more infected all birds inoculated. No variation in susceptibility was apparent at different seasons of the year.

Five of 40 birds which became infected survived. The amounts of virus which survivors had received ranged from 0.4 to 5.6 LD_{50} units. Infected individuals survived in July, September, November, and February. Amounts of virus in excess of 7.6 LD_{50} units caused the death of all of 10 birds thus exposed.

Deaths considered due to infection occurred between the second and ninth days after inoculation, the majority occurring between the second and fifth days. The death rate was highest in those groups which received the largest dosage of virus.

Virus was present in the blood of 31 of 40 infected birds within 17 hours after inocula-

tion; in 35 of 40, maximum titers were reached on the second day. In those cases where death did not intervene, blood virus titers remained above $10^{4.0}$ LD_{50} for 2 days in 8 of 22 individual birds and for 3 days in 14 of 22. After the second or third day, blood virus titers gradually declined and the virus disappeared by the fifth or sixth day. In general, viremias were similar in titer and duration regardless of the size of the inoculum, or the month of the year in which the birds were infected.

To check for prolonged incubation periods or recurrence of viremia, the surviving birds of the group inoculated October 27, 1953 were bled daily for 2 weeks. No virus was detected in any sample obtained after the fourth day following inoculation.

Preinoculation and final blood samples from the surviving birds were tested for neutralizing antibody. None of the birds showed evidence of immunity before inoculation. Only those birds in which viremia was evident developed antibody. Neutralizing antibody was detected as early as 6 days after inoculation, reached maximum levels between 21 and 84 days, and was still present 109 days after inoculation.

Discussion. The high susceptibility and blood virus titers observed in these studies support previous evidence that the English sparrow has a high potential as a source of EEE virus for infecting mosquitoes(7). However, this potential may be somewhat decreased by the high mortality rate early in the course of the viremia. The finding that only birds which developed detectable viremia subsequently developed measurable antibody is in contrast to the picture in horses in which neutralizing antibody may develop in the absence of detectable viremia(8). These studies did not indicate that susceptibility, viremia levels attained, or mortality were influenced by the age, sex, or reproductive status of the bird or by the season of the year.

Summary. 1. Eight groups of wild caught English sparrows totaling 78 individuals were inoculated with eastern equine encephalomyelitis virus from July 1953 to April 1954. 2. No significant differences in response to infection were observed which could be related to age, sex, reproductive status or season. 3. Information on infective dosage, blood virus levels developed, mortality, and development of neutralizing antibody is presented.

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Prevention of Acute Respiratory Illness in Recruits by Adenovirus* (RI-APC-ARD) Vaccine.† (22484)

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Acute respiratory illness caused by viruses of the RI(1,2) (also called APC(3) or ARD (4)) family is a major medical problem to the Armed Forces. In certain recruit training camps, these viruses have been found responsible for as many as 90% of the hospital admissions for respiratory disease during the winter months(5-8) and for up to 60% of all hospital admissions for these illnesses during the entire year(9). Respiratory illnesses caused by agents of the RI group are included in the general disease category of the febrile catarrhs(10) but belong, more specifically, among the entities of undifferentiated acute respiratory disease (ARD)(11), non-streptococcal exudative pharyngitis(12), primary atypical pneumonia (PAP) unassociated with cold agglutinins(13), bronchitis resembling atypical pneumonia (Br-AP)(14) and pharyngo-conjunctival fever(15). The viruses of the RI family comprise at least 14 distinct serotypes(2,3,8,16,17) but only 3 of these, types 3, 4 and 7, have been found important in the causation of respiratory illness of soldiers, types 4 and 7 being most frequent(2,8, 18). Patients with RI virus infections develop both neutralizing and complement-fixing antibodies against the infecting virus(1). The neutralizing antibody appears to be associated with protection against the disease (4,19), while complement-fixing antibody is directed against a "soluble" group-specific antigen(2,3,20) which is common to the agents of the RI group and appears not to be protective(5,7).

The impact of RI-caused illness on the training of recruits in the Armed Forces makes highly important the development of a vaccine which will protect against the natural disease under field conditions. Huebner, *et al.* (19) have recently reported the effectiveness of a type 3 killed-virus vaccine in the prevention of an experimentally induced disease in volunteers challenged by inoculation of the homologous virus onto the conjunctivae. The present report describes the development, in our laboratory, of a bivalent formalin-killed types 4 and 7 vaccine prepared from infected monkey kidney tissue cultures. This was found highly effective, under field conditions, in reducing the incidence of acute respiratory illness of RI etiology in recruits.

Materials and methods. Vaccine preparation. The RI virus strains used to prepare the vaccine were: RI-67(1), a type 4 virus originally recovered in human tracheal epithelium tissue culture from the throat washing of a case of PAP; and a type 7 virus, RI-4-202(2), recovered in human amniotic epithelium tissue culture from a throat washing of a case of ARD. These strains were adapted from the original human cell isolations to monkey kidney epithelial culture¶ maintained in serum-free synthetic mixture 199¶ containing 200 units of penicillin and 200 μg of streptomycin per ml. The infected whole cultures were harvested 4 to 7 days after viral inoculation, homogenized in a Waring blendor, clarified by spinning at 2500 rpm for 20 minutes in a horizontal centrifuge, and filtered through a sintered glass filter of medium porosity. These filtrates, after neutralization

* Viruses previously called RI, APC or ARD agents have recently been named "Adenoviruses."

† This study was carried out at Fort Dix, N. J. using facilities of U. S. Army Hospital, Col. R. H. Eckhardt, Commanding; Fort Dix Health Center, Lt. Col. J. Cooch, Commanding; and Influenza Vaccine Evaluation Program of Commission on Influenza, Armed Forces Epidemiological Board, Dr. Harry Rose, Director.

¶ Tissue cultures and tissue culture fluids employed were obtained from Microbiological Associates, Bethesda, Md.

with 0.5 M monobasic sodium phosphate solution, had infectivity titers of 10^{-1} or 10^{-2} in HeLa cell cultures, complement-fixation (CF) titers of 1:2 or 1:4 and protein nitrogen values between 0.009 and 0.016 mg/ml. Prior to inactivation, the identity of each filtered virus lot was proved by serum neutralization tests performed with monotypic rabbit antisera. The failure of the mixtures of virus and homotypic serum to produce degeneration in monkey renal epithelium and HeLa† cell cultures identified the virus and showed the absence of other detectable cytopathogenic agents. To prepare vaccine, the filtered virus suspensions were treated with freshly redistilled formalin (assay 35.1%) to a final concentration of 1:4000 and held at 36°C in a water bath with occasional shaking for 6 days. This inactivation period was twice that required to render the virus non-infectious. Twenty ml aliquots of the formalin-treated filtrate were dialyzed at 4°C against Hank-Simms† solution to remove free formaldehyde. This same procedure performed on live virus preparations did not cause measurable reduction in the infectivity titers. To test for completeness of formalin inactivation, 10 ml amounts of the dialyzed vaccine were inoculated in duplicate into 32 oz flask cultures of renal epithelium maintained in mixture 199. These inocula did not induce significant cytopathogenic change during incubation at 36°C for 12 days nor did extracts of the cells from these cultures cause degeneration when passed to tube cultures of monkey kidney and observed for an additional 12-day period. For use in human beings, two bivalent pools (Lots 1 and 2) consisting of equal volumes of type 4 and type 7 formalin-inactivated vaccine were prepared, distributed in 20 ml amounts into rubber-stoppered dispensing bottles and stored at 4°C until used in the field trial.

Safety and sterility tests. The first series of safety tests was carried out on the virus preparations prior to the addition of formalin. Hamsters were inoculated intraperitoneally; suckling mice intracerebrally and intraperitoneally; adult mice intracerebrally; rabbits intraperitoneally, intracerebrally, and intracutaneously; guinea pigs intraperitoneally; and rhesus monkeys intracerebrally and intramuscularly. These animals were observed for periods of 21 to 42 days during which times there were no deaths and no fever or other signs of illness. No gross pathological lesions were noted at autopsy nor did Leptospirae grow from cultures of hamster blood. The monkeys did not develop detectable neutralizing antibody against Types I, II or III poliomyelitis virus, nor did they show evidence of poliomyelitis on histopathological examination‡ of the brain and cord. The virus preparations did not show detectable growth when cultured aerobically and anaerobically at 37°C and at room temperature on a variety of media§ chosen to detect fungi and bacteria, including *Mycobacterium, Brucella, Pasteurella,* pleuropneumonia and other human pathogens. The final safety and sterility tests on the prepared bivalent vaccine pools consisted of inoculating one ml of the vaccine into 400 ml portions of thioglycollate broth in triplicate and intraperitoneal inoculation of guinea pigs and mice. None of these tests showed any detectable microorganisms. *Virus laboratory tests. Virus recovery* attempts from patients' throat washings were carried out in duplicate in HeLa cell cultures and in the Henle strain of human intestine cells† by the same general methods described earlier(1). Passage of the triturated whole cultures was made at 3- to 7-day intervals and from 3 to 5 such serial passages were made before any specimen was discarded as negative. The recovered viruses were identified as agents of the RI family by demonstration of the presence of RI group CF antigen in the triturated whole culture and the strains were typed by methods already described(1) according to the classification system of Rowe, *et al.*(3).

‡ Histopathological examinations were made by Dr. J. E. Smadel.

§ These included Sabouraud's agar, trypticase soy agar, tryptose agar, blood agar, serum ascites agar, Dubos Tween albumin medium, Lowenstein's medium, egg-meat infusion, brain-heart infusion with 0.1% agar, and Brewer's thioglycollate broth. We are indebted to Miss S. Carey of the Dept. of Bacteriology, Walter Reed Army Institute of Research, for performing these tests.

TABLE I. Serological Response in Guinea Pigs to Monovalent Killed RI Virus Vaccines.

Vaccine		Homologous neutralizing antibody titer*		RI group, CF antibody titer*	
Virus type	Pool No.	Aqueous	Adjuvant	Aqueous	Adjuvant
4	1	2	32	0	20
	2	2	32	0	10
7	3	0†	32	0	0

* Titers are expressed as reciprocal of serum dilution.

† 0 titer value in the neutralization test equals less than 1:2 and in the CF test less than 1:5, the lowest serum dilutions tested.

Serology. The methods for the CF and neutralization tests were described previously(1). All serum titers are expressed as the greatest initial dilution of serum which caused complete or nearly complete suppression of virus growth (neutralization) or of fixation of complement.

Results. Neutralizing and CF antibody response to vaccination in guinea pigs and volunteers. Groups of six 800 to 1000 g guinea pigs were given one intramuscular injection of 0.25 ml of inactivated aqueous monovalent vaccine or 0.50 ml of an adjuvant-vaccine emulsion consisting of 0.25 ml of the vaccine and 0.25 ml of Arlacel in mineral oil (10% Arlacel, 90% Drakeol) mixture.‖ The animals were bled 4 weeks post-inoculation and pooled aliquots of the sera tested for content of homologous neutralizing and RI group CF antibodies. As shown in Table I, the neutralizing antibody titer resulting from injection of type 4 aqueous vaccine was 1:2 and from type 7 vaccine was below the detectable level. The neutralizing antibody response to all adjuvant preparations was 1:32. The only vaccine which stimulated CF antibody production was Type 4 in adjuvant.

To test the vaccine in man, each of 12 volunteers, military or civilian personnel of the Walter Reed Army Institute of Research, were given 1 ml of the bivalent preparation into the triceps muscle. Table II, which shows the CF and neutralizing antibody titers of the individual sera from 5 volunteers collected prior to and 3 weeks post-vaccination, illustrates the variety of antibody response in the group as a whole. It is seen in the table that nearly all persons developed an increased amount of neutralizing antibody against not only homotypic 4 and 7 virus but heterotype 3 as well. These increases in titer were as great as ordinarily obtained following the natural disease(1,5,6). Volunteers 1, 2 and 3, who received 2 additional injections of the vaccine at 3- and 5-week intervals, failed to show any further increase in neutralizing antibody. In no instance has repeated injection been found superior to a single injection alone. Measurable CF antibody response was very infrequent and of low titer as illustrated by volunteer 2 in Table II.

Field Evaluation. Study population. The clinical evaluation of the vaccine was made at Fort Dix, N. J., an Army post where recruits are given basic training. At this post, the RI virus infection rates have been consistently high during the winter months in recent years(5,7). *Organization of the study.* At the time this study was undertaken, an influenza vaccine evaluation program was being conducted at Fort Dix by the Commission on Influenza, Armed Forces Epidemiological

‖ Supplied by Dr. I. W. McLean, Parke-Davis and Co., Detroit, Mich.

TABLE II. Serological Response in 5 Volunteers to Bivalent Types 4 and 7 Killed RI Virus Vaccine.

Serum specimen	Neutralizing antibody titer*			RI group CF antibody titer*
	Homotype 4	Homotype 7	Heterotype 3	
Pre-vac.†	0‡	2	0	0‡
3 wk post-vac.	32	32	2	0
Pre-vac.	2	0	0	0
3 wk post-vac.	8	8	8	5
Pre-vac.	2	2	2	0
3 wk post-vac.	2	8	8	0
Pre-vac.	0	0	8	0
3 wk post-vac.	8	128	128	0
Pre-vac.	0	0	0	0
3 wk post-vac.	32	32	2	0

* Titers are expressed as reciprocal of serum dilution.

† Pre-vac. = Pre-vaccination. Post-vac. = post-vaccination.

‡ 0 titer value in the neutralization test equals less than 1:2 and in the CF test less than 1:5, the lowest serum dilutions tested.

Board, under the direction of Dr. Harry Rose. In this program, influenza vaccine was given to the first 100 men assigned to each company with the remaining 100 to 150 men of the unit being retained as controls. By arrangement with Dr. Rose, the control groups of all 6 companies which began basic training between 20 February and 5 March 1956, were made available for the evaluation of the RI virus vaccine. These were divided into two groups by the following procedure. As rosters were received assigning the men to a company, case numbers were given in sequence to the men entering the study. During the precycle week, a team of technicians visited each unit, drew a prevaccination blood sample and gave the initial inoculation into the triceps muscle. In the first company, recruits whose case numbers were odd received 1 ml of the RI vaccine (Lot 1) and those whose case numbers were even were given a formalin-saline placebo and retained as controls. In the second company, the procedure was reversed and the even-numbered men received the vaccine and the odd-numbered men the placebo. This alternation of odd and even numbers by company was carried out until all 6 companies were vaccinated. A 1 ml dose of Lot 2 vaccine or placebo was given approximately 7 days following the first injection. In all, 311 soldiers received vaccine while 313 received placebo. Additional specimens were drawn from all soldiers in the study group at some time during the third and seventh week of training. Complete records were kept of all hospital and dispensary admissions from the 6 companies under study. All recruits admitted to the hospital with a diagnosis of any acute respiratory condition were bled at the time of admission and again approximately 3 weeks later. In addition, a throat washing for virus recovery was collected in nutrient broth from the patients while in the admitting room. These were frozen immediately in dry ice in tightly sealed screw-cap jars and transmitted to the laboratory at the Walter Reed Army Institute of Research where they were maintained in dry ice until tested 3 to 10 weeks later. All blood specimens were collected in vacuum venules and transmitted to the same laboratory where the serum was separated from the clot and stored at 4°C until tested 2 to 3 months later.

TABLE III. Total Hospital Admissions for Respiratory Illness and Admission Rates in Vaccinated and Control Groups.

Inoculum	No. of persons in group	No. of hospitalized cases from group	Admission rate in %
Cases occurring during entire period of observation:			
RI vaccine	311	32	10.3
Formalin-saline control	313	89	28.4
Cases occurring 7 days or more following initial inoculation:			
RI vaccine	293	14	4.8
Formalin-saline control	294	70	23.8

Clinical evaluation of RI vaccine effectiveness. The numbers and percentages of soldiers hospitalized from acute respiratory illness in the 311 vaccinated and 313 control recruits are summarized in Table III. The overall figures for the entire period of observation are not particularly impressive since 10.3% of the soldiers receiving vaccine were hospitalized compared with 28.4% of those given placebo. However, when the cases occurring during the first week post-vaccination are excluded, the findings are dramatic. Only 4.8% of the vaccinated were hospitalized compared with 23.8% of the controls. The importance of considering the interval between vaccination and onset of illness is illustrated in Fig. 1 which shows the admissions for each week from time of first vaccination. Essentially the same number of cases were hospitalized in each group during the prevaccination period and first week following the initial dose. However, during the 2nd, 3rd and 4th weeks, about 11 times as many cases developed in the control group as occurred among an equal number of vaccinated soldiers. During the last 4 weeks of basic training, at a time when the susceptibles were largely depleted, very few cases of respiratory illness occurred and no difference was apparent in the incidence in the two groups. The beneficial effect of vaccination, therefore, did not appear until one week after the initial vaccination and disap-

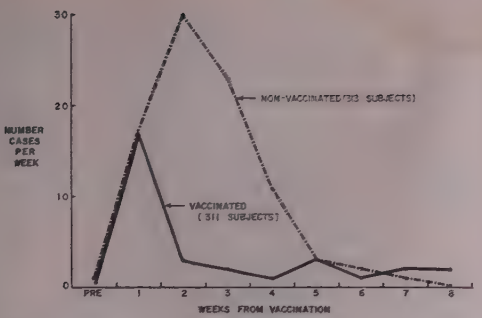

FIG. 1. No. of hospital admissions for acute respiratory illness—vaccinated and control subjects by week from vaccination. Fort Dix, N. J., winter, 1956.

peared during the last 4 weeks.

Laboratory findings. The results of the virus isolation and serological tests showed that approximately 9 of every 10 cases of acute respiratory illness admitted to the hospital during the study period were of RI etiology showing that the vaccine was tested during the course of an epidemic of RI virus infection. Forty-nine RI viruses were recovered from throat washings from 65 patients hospitalized for acute respiratory illness. Virus was recovered from 4 of 12 (33%) vaccinated individuals and 37 of 49 (75%) non-vaccinated persons. Among 41 RI virus isolates typed to date, 24 (59%) were type 7 and 17 (41%) type 4. The percentage distributions of types within the vaccinated and non-vaccinated groups were essentially the same. Acute and convalescent sera from 97 patients, tested for amount of RI CF antibody, revealed a diagnostic (4-fold or greater) increase in antibody titer in 78 which was related temporally to the clinical illness. Eighteen of 28 (64%) vaccinated persons and 60 of 69 (87%) non-vaccinated individuals showed such increase in antibody.

Discussion. The relatively low infectivity titers, 10^{-1} or 10^{-2}, of the virus preparations prior to inactivation would ordinarily suggest insufficient quantity of antigen to permit its use as a vaccine without virus concentration. However, the infectivity titers of such preparations do not reflect the true content of virus. Electron microscopy has shown virus particle counts of 10^{9} in similar preparations which titered 10^{-2} or 10^{-3}(20) indicating the presence of amounts of virus material which might reasonably be expected to be immunogenic. The first confirmation of this hypothesis was the demonstration of detectable neutralizing antibody levels in sera of guinea pigs following injection of aqueous or adjuvant preparations. The neutralizing antibody response in guinea pigs to the aqueous vaccine was low, as might be expected in animals with no previous experience with these antigens. The response in man, however, was quite different since relatively high levels of antibody were readily achieved with this vaccine. Further experience(9) has shown that maximal or near maximal levels of neutralizing antibody are achieved within 1 week after a single injection of vaccine. This, probably, is a manifestation of the recall phenomenon in persons with previous experience with RI antigens common to types 4 and 7, if not the result of earlier infection with types 4 and 7 viruses themselves. The fact that additional injections of vaccine after the first failed to increase the antibody level is consistent with the hypothesis that the first dose of vaccine acts to elicit a recall type of response.

The vaccine has now been given to approximately 350 persons, in single or multiple doses, without untoward effect. The low protein content of the vaccine, which is in the range of that of the current poliomyelitis vaccine, suggests that the production of sensitivity to monkey kidney component is not likely to be a problem.

The administration of this RI virus vaccine to newly inducted recruits effected a striking reduction in the incidence of hospitalized cases of acute respiratory illness. Although no beneficial effect was apparent during the first week after vaccination, the reduction of cases beginning in the second week and continuing in the third and fourth weeks post-vaccination was dramatic. This interval corresponds to the time when the vast majority of cases of RI disease have been shown to occur in recruit camps(7). During this period of high incidence, 64 cases occurred among 313 placebo-inoculated troops compared with only 6 among the 311 who received the RI

vaccine. After the fourth week, the incidence fell to low levels in both vaccinated and control groups. This pattern conforms to previous experience in recruit camps(7) and would seem to indicate the emergence of respiratory illnesses of non-RI etiology in troops among whom the RI susceptibles have been reduced to a low level. This hypothesis is strengthened by the fact that most of the late cases tested to date have been shown to be of non-RI etiology. It would appear that one dose of vaccine is sufficient to protect since, as described above, it acts as a recall mechanism. Hence, the second dose, as given in this study, is probably unnecessary. The failure of the vaccine to protect during the first week was expected and can be explained readily when one adds the known 4- to 5-day incubation period of RI virus disease to the 3 or 4 days necessary for recall antibody to develop. No information was obtained with respect to the duration of the immunity gained as a result of RI vaccination. The disappearance of RI illnesses from these companies after the fourth week rendered it impossible to study this question.

It is of interest to note that the pattern of cases of acute respiratory disease among the recruits who received influenza vaccine paralleled that of the control group throughout the training period. These men did not form part of the study group but nonetheless may be used as a secondary control to confirm the efficacy of RI vaccine.

As has been noted, the initial dose of vaccine was given after the men were assigned to their training companies and after cases of RI virus disease had already begun to occur in the men. A more marked overall reduction in the incidence could be expected if the vaccine were administered at the time the recruit is first inducted into the Army or as soon as possible after his arrival at the training base. By doing this, the incidence could be reduced in the first week of the training cycle.

It should also be noted that this trial was conducted during a season when little influenza was present on the base. The total percentage reduction in the incidence of acute respiratory illness brought about by RI vaccine will, of course, vary greatly depending upon the current incidence of influenza and other respiratory conditions of non-RI virus etiology.

Summary. The development, preparation and field evaluation of a highly effective formalin-killed vaccine against acute respiratory illnesses caused by viruses of the RI (Adenovirus) family has been described. The vaccine, prepared from infected tissue cultures of monkey renal epithelium, contained types 4 and 7 virus and was not injurious upon human injection. In human volunteers given a single 1 ml dose of vaccine intramuscularly, it induced high levels of neutralizing antibody. In 311 recruits, vaccinated at Fort Dix, N. J., it produced a marked reduction in the incidence of acute respiratory illness beginning one week after the initial dose of vaccine; only 4.8% of those receiving RI vaccine were hospitalized for respiratory illness compared with 23.8% of the control group of 313 which received placebo.

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Effects of Mescaline in Laboratory Animals and Influence of Ataraxics on Mescaline-Response. (22485)

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Mescaline has been of continuing interest to biologists because of its ability to produce "model psychosis" in man(1) as well as experimental catatonia in laboratory animals (2). Current interest has been stimulated by the findings that α-(4-piperidyl)benzhydrol HCl (azacyclonol, Frenquel) antagonizes mescaline-induced hallucinosis in man(3,4), electroencephalographic changes in man(5) and rabbits(6), and spasm of isolated estrus-rat uteri(7). Because of preliminary clinical reports on the beneficial effects of azacyclonol in mentally-disturbed patients, this drug has been included in a category of drugs known as "ataraxics" or "tranquilizers" together with reserpine and chlorpromazine(4). The present study was undertaken, first, to determine whether or not reasonably objective indications of behavioral response could be obtained following mescaline in laboratory animals and, second, to study the ability of ataractic compounds to antagonize the effects of mescaline on behavior.

Materials and methods. Mescaline sulfate was administered to dogs and cats intravenously, intraperitoneally, and into the lateral ventricle of the brain after the method of Feldberg and Sherwood(8,9). Permanent intraventricular cannulae of stainless steel were implanted in the cranium and covered with a skin-flap, placement being confirmed by x-ray or dissection following death or sacrifice. These cats showed no apparent ill effects from the cannulae. Routinely, antibiotics (500 mg dihydrostreptomycin and 100,000 U penicillin G, intramuscularly) were administered for 3 days after operation, as well as the day of and day after each intraventricular experiment. These experiments were not begun until at least one week after operation and the cats were rested 5-10 days between tests. Drugs were administered intraventricularly in volumes of 0.5 ml or less under sterile conditions, with the cats held under minimal restraint. The volume of the cannulae was approximately 0.1 ml.

Results. Intravenous mescaline in dogs. In 6 experiments on 4 dogs, 20 mg/kg of mescaline caused repeated defecation and urination, tachypnea, salivation, mydriasis, and apparent dizziness. These effects lasted 10-20 minutes and were succeeded by a 4-hour period during which the dogs lay or stood nearly motionless in one place, exhibiting marked disregard for and non-reactivity to visual, auditory, and pain stimuli. This condition resembled that in cats described by de Jong(2), which he termed "catatonia," as well as that recently described in dogs by Hosko and Tislow(10); it also duplicated some of the effects of mescaline in man, such as salivation, emesis, mydriasis, immobility, and decreased reactivity to pain(2,11,12). When the dogs were forced to move, they

stepped slowly and gingerly, but without signs of ataxia or hypotonia. There was no apparent tolerance in 2 dogs on whom the experiment was repeated 24-48 hours later. Three of the dogs were subsequently pretreated with 50 mg/kg azacyclonol and then given mescaline 1 hour later. Azacyclonol caused no gross effects itself, but after mescaline the dogs became quiet, one of them vomiting and panting, and another urinating; all were completely normal in 5-15 minutes. It appeared that the catatonic response to mescaline had been prevented by azacyclonol.

Intraperitoneal mescaline in cats. For comparison, the effect of 50-75 mg/kg mescaline was observed in 6 experiments on 4 cats. They developed autonomic signs (salivation, emesis, tachypnea, mydriasis) and behavioral signs (progressive depression leading to catatonia). The latter resembled the "catalepsy" and "negativism" in cats described by de Jong (2) following doses of more than 25 mg/kg intramuscularly.

Intraventricular mescaline in cats. After the foregoing initial studies, it was decided to restrict experimentation to drugs administered by this route, primarily to concentrate on reactions in the central nervous system. Mescaline was injected 21 times in 18 cats in doses ranging from 1-3 mg in 0.5 ml sterile 0.5% saline. Injection of saline alone caused no appreciable effect. Immediately after mescaline, the cats began a loud, continuous yowling that was unlike any normal cat-sound and that could usually be precipitated, if it did not begin spontaneously, by a sudden, loud noise. During the next 20 minutes, there was in addition a succession of salivation, tachypnea, retching, lacrimation, defecation, urination, and mydriasis. Of these signs, yowling, salivation, tachypnea, retching, and defecation occurred in every cat; the remainder occurred in the majority. The facial expressions were very similar to those depicted by de Jong(2). The cats displayed a decreasing interest in the environment and could be stimulated to move only with difficulty ("negativism"); this condition began to develop at about 10 minutes and lasted for 4 or more hours. Body movements were slowed and somewhat ataxic when the cats were forced to jump or walk; following this, they quickly resumed their stuporous immobility. This depressed state was more severe than the ataraxia produced by intramuscular chlorpromazine (20 mg/kg) or intraperitoneal reserpine (0.5 mg/kg) in cats, and again corresponded more closely to the syndrome of "decreased motor initiative, negativism, and catalepsy"(2). There was, however, no evidence of complete plastic rigidity, as is commonly caused by bulbocapnine (2,13). These autonomic and psychomotor effects of mescaline will be referred to below as the "mescaline-response."

Five cats that instantaneously caught, killed, and ate a mouse several hours before intraventricular mescaline were each offered another mouse 30 minutes afterwards. The cats at first ignored the mice, one of them even submitting placidly to having his ears and nose nibbled. Later, the cats began to regard them with curiosity; they appeared to derive enjoyment from rubbing their cheeks against the mice and allowing them to crawl over and under their bodies. This behavior was interrupted by periods of immobility and persisted until some 18-20 hours later (only 3 hours in 1 cat), at which time the "fondling" became progressively more rough, ending with a typical "cat-and-mouse" play in which the mice were killed and eaten. Presumably, this progression in behavior was associated with clearance of the drug. A sixth cat, which normally was not a "mouser," displayed similar "fondling" behavior towards mice while under the influence of mescaline; moreover, his normally antagonistic attitude towards human beings was softened into a playful tractability and friendliness. The failure of catatonic cats to attack mice was lucidly depicted by de Jong(2) for a case of acetylcholine-catatonia.

It is noteworthy that the paroxysms of scratching noted by Schwartz and co-workers in 3 cats(30) were not observed in any appreciable number of cats we have injected with mescaline to date. The reason for this discrepancy is not at once apparent.

Reserpine and the mescaline-response. In this and the following experiments, cats were

used that had previously been standardized on mescaline. The immediate effects of reserpine (0.1 mg intraventricularly in 4 cats) were variable and included 2 cases each of meowing as if in pain, mydriasis, defecation, and mild ataraxia or drowsiness and indifference. One cat had generalized tremors and appeared dizzy; another cat salivated. These effects were mild and the cats seemed completely normal 2 hours later. It is noteworthy that these cats had not yet displayed the well-known signs that always follow 0.05-0.1 mg/kg reserpine given intraperitoneally: miosis, nictitating membrane relaxation, and an ataractic state approaching the stupor of catatonia.

Approximately 2 hours after intraventricular reserpine, 2 mg mescaline were administered similarly. The cats developed the immediate symptoms of mescaline intoxication (yowling, tachypnea, defecation), but failed to progress into a state of catatonia. By 5 hours after injection, the cats were developing miosis and were somewhat less active than usual, *i.e.*, mildly tranquil. The following day they showed the typical residua that result from reserpine: miosis, nictitating membrane relaxation, anorexia, diarrhea, and tranquility or ataraxia. This experiment suggested that reserpine pretreatment antagonized the development of mescaline-catatonia.

Chlorpromazine and the mescaline response. Chlorpromazine (2 mg intraventricularly in 2 cats) caused nictitating membrane relaxation, tachypnea, ataxia, and a marked catatonia, wherein the cats appeared oblivious of visual, auditory, and pain stimuli and maintained abnormal positions (catalepsy). Approximately 2½ hours later, 2 mg mescaline were injected similarly. The only discernible change was a deepening of the catatonia and a dilatation of the pupils. Since these cats were already catatonic when mescaline was administered, the results from mescaline were inconclusive. Therefore, the experiment was repeated in 4 additional cats injected with 1 mg chlorpromazine. This dose level quieted all 4 cats and caused mild ataxia and nictitating membrane relaxation in 2; all were completely normal 2-3 hours later when 2 mg mescaline were administered. The only response to mescaline observed was a moderate decrease in spontaneous activity. This second experiment suggested that pretreatment with doses of chlorpromazine, which in themselves caused no appreciable effect, almost completely blocked the mescaline-response. The effects of higher doses of chlorpromazine given intraventricularly were similar, but not identical, to those following intramuscular injection (5-20 mg/kg): nictitating membrane relaxation, moderate miosis, marked ataxia, and a non-cataleptic state of ataraxia.

Azacyclonol and mescaline-response. Three cats received 4 injections of 5 mg each of azacyclonol intraventricularly and 2 received 1 mg. The first 3 cats responded with tachypnea, emesis, tremors, nictitating membrane relaxation, ataxia, and a moderate degree of ataraxia. The latter 2 cats responded with tachypnea, ataraxia, and in 1 case emesis. In no case was there evidence of hyperkinesis, as produced by high parenteral doses (14). All of the cats were substantially normal except for residual ataxia when injected 2½ hours later with 2-3 mg mescaline intraventricularly. None of the autonomic responses to mescaline was blocked (see 3,6), but the psychomotor responses were altered from a catatonic to an excitatory type in 4 of the 5 cases ("crying," tremors, attacking, hissing, running, restlessness, and in 1 cat convulsions). This state of excitation passed into one of ataraxia in 1 cat, resembling that seen in the cat that failed to show any excitation at all. Another test was modeled after Fabing's experiment on the blockade of d-lysergic acid diethylamide (LSD)-psychosis in man by azacyclonol prophylaxis(3). Two cats received 5 mg azacyclonol intramuscularly each day for 5 days and, on the 6th day, a final 5 mg 2 hours before the intraventricular injection of mescaline. One cat displayed the typical yowling, mydriasis, tachypnea, and catatonia for 80 minutes after 2 mg mescaline; the other cat panted, defecated, and remained ataractic for 30-45 minutes after 1 mg. Both were completely normal 2 and 1 hours later, respectively, suggesting a decreased duration of response to intraventricu-

lar mescaline following chronic azacyclonol prophylaxis.

Discussion. In dogs and cats, mescaline injected intravenously or intraperitoneally produced a prolonged period of catatonic immobility that was apparently prevented in the former by pretreatment with azacyclonol. When injected into the lateral ventricle of the cat's brain, mescaline caused a characteristic yowling, various autonomic effects, and catatonia. It has been our experience that a certain degree of tolerance is developed by cats injected every 10-14 days with mescaline: the minimal effective dose for marked catatonia increased from approximately 1.5 to 2.5 or 3.0 mg. This did not appear to be true for the convulsive dose, however, which remained slightly over 3.0 mg. Pretreatment intraventricularly with ataraxics had the following apparent influences on the mescaline-response: reserpine antagonized the development of catatonia, chlorpromazine almost completely inhibited all of the responses to mescaline, and azacyclonol tended to change the catatonic mescaline-response into an excitatory type. The reactions of cats to intraventricular reserpine and azacyclonol alone differed from those typically appearing after intraperitoneal or intravenous administration, while the reactions to chlorpromazine were similar. Moreover, the effects of intraventricular mescaline were different from those of intraventricular LSD(15,30) and the effects of intraperitoneal or intramuscular reserpine were different from those of intraventricular 5-hydroxytryptamine (serotonin)(16,30).

A certain amount of care should be exercised in the interpretation of the foregoing experimental results and conclusions, since the observation and classification of behavioral responses were always necessarily subjective. At high dose levels, we were not at all certain that a clear distinction could be made, for example, between catatonic stupor and marked ataraxia. Therefore, we attempted to use doses of ataraxics that were low enough to render the cats essentially normal by the time mescaline was administered.

The mescaline-blocking action of chlorpromazine suggested by these studies is consistent with previous findings that this drug antagonized the psychotic effects of mescaline and LSD in human volunteers(11,17,18) and the electroencephalographic changes caused by LSD(19). The results with azacyclonol are consistent with the reports that it antagonized hallucinogen-induced psychoses(3,4), electroencephalographic changes(5,6), and *in vitro*-uterospasm(7). Moreover, all 3 ataraxics have shown promise in the therapy of mental illness.

It is noteworthy that the 3 ataraxics employed in this study are serotonin-blockers, while small amounts of mescaline and LSD are serotonin-potentiators(20). This suggested to Costa(20) that, in general, hallucinogens may potentiate and ataraxics may antagonize serotonin in the brain, thereby producing their characteristic psychic effects. Dosage, however, is an important consideration, for it is well-known that larger amounts of LSD block serotonin(20). The situation is further complicated by the facts that large amounts of serotonin and reserpine, which releases bound - serotonin(21-23), potentiate hexobarbital-hypnosis in mice and that this potentiation is reversible by huge doses of LSD(21,24), but not by mescaline(25). Hexobarbital-potentiation is also a property of iproniazid(25), a compound that prevents the destruction of serotonin by monamine oxidase(26). Although chlorpromazine is also a potentiator(27), it does not appear to be a serotonin-releaser(28); moreover, the potentiation is not reversible by LSD(29). Azacyclonol was reported to potentiate hexobarbital in mice(14), but neither Shore(29) nor we (unpubl. data) have been able to confirm such activity at moderate dose-levels. The foregoing observations emphasize certain differences among the ataraxics, as well as between mescaline and LSD. These considerations, among others, suggest that these ataraxics may have different mechanisms of psychic action.

Summary. Mescaline, injected intravenously and intraperitoneally into dogs and cats and into the lateral ventricle of the brain of cats, produced acute autonomic effects followed by catatonia. These responses ap-

peared to be altered or prevented by pretreatment with the ataraxic agents chlorpromazine, reserpine, and azacyclonol.

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Similarity of Effect of "Adrenalin", Adrenaline and Nor-Adrenaline on the Cat Denervated Heart.* (22486)

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In view of the current interest in and conflict of evidence as to possible similarity or difference in action of adrenaline and noradrenaline, these have been tested as to their effect on the acutely surgically denervated heart of the cat. This report describes the chronotropic effect of extracted glandular adrenaline and of synthesized l-adrenaline and l-nor-adrenaline on this preparation.

Methods. Fasted cats, males and females, were anesthetized with Dial-Urethane (Ciba), 0.6 cc/kilo of body weight injected intraperitoneally. The heart was denervated by vagal section and removal of the stellate ganglia as first described by Levy(1); blood pressure and heart rate were recorded by mercury manometer from the left carotid artery; injec-

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TABLE I. Summary of Observations on All Animals Studied.

Dose level (μg/kg/min.)	"Adrenalin"				Adrenaline				Nor-adrenaline			
	No. observations	Avg total change*	S.D.†	S.E.M.‡	No. observations	Avg total change	S.D.	S.E.M.	No. observations	Avg total change	S.D.	S.E.M.
.25									10	51	30	9
.5	29	111	52	10					7	117	43	16
1.0	30	193	63	11	17	266	120	29	10	177	81	26
1.5	31	279	74	13								
2.0	30	338	100	18					9	289	141	47
2.5	29	361	87	16								
3.0	28	390	131	25	16	430	140	37	8	396	216	76
6.0	31	573	106	19	20	569	154	34	6	481	249	102

* Avg total change in No. of heart beats for 10 min. test period. † Stand. dev. ‡ Stand. error of mean.

tion was by way of a cannula in the right saphenous vein. *Adrenaline* and *nor-adrenaline* were tested in the following preparations: (1) gland extract as commercial Adrenalin Chloride of Parke, Davis and Co. which will be referred to as "Adrenalin"; (2) synthesized l-adrenaline, the l-epinephrine d-bitartrate, control no. R-041-EK of Sterling-Winthrop Research Institute which will be referred to as adrenaline; and (3) l-nor-adrenaline, Levophed, l-arterenol d-bitartrate monohydrate of Winthrop-Stearns Inc. which will be called nor-adrenaline. Injections of freshly prepared solutions were given at a constant rate of 1 cc per minute for 5 minutes. A preinjection record was obtained just prior to each infusion with subsequent records taken of the last 10 seconds of each minute during the injection and of each of the 5 minutes immediately following injection. Not less than 25 minutes were allowed between each infusion. Doses were calculated as micrograms of base substance per kilo of body weight per cc injected per minute, the dilutions being made in isotonic saline solution. "Adrenalin" response was taken more or less as the general frame of reference for comparison and consequently was tested over a more complete dosage range than the other 2, as is shown in Table I which gives the doses used for each drug expressed as micrograms per kilo of body weight per cc injected.

Results. Table I also gives the total chronotropic change for the 10-minute period including the 5 minutes of injection and the subsequent 5 minutes recovery. It may be said that the effects during the 5-minute injection period only are, of course, of less magnitude but of exactly similar relative value. The response of the denervated heart of the cat to these 3 preparations is remarkably similar (Table I and Fig. 1). This is especially true within the physiological range between 0.25 and 3.00 μg per kilo per minute and at this latter dosage, presumably about the upper limit of the secretatory capacity of the adrenal medulla for sympathomimetic amines the similarity is complete (Fig. 2).

Discussion. When Goldenberg, *et al.*(2) studied the hemodynamic response to adren-

aline and nor-adrenaline in man, they claimed the 2 substances had opposite effects. Adrenaline acted as an over-all vasodilator and a powerful cardiac stimulant, increasing the rate and output while nor-adrenaline was an intense vasoconstrictor, slowing the heart and decreasing the cardiac output. This implies distinct qualitative differences not previously observed. There has been considerable reiteration of these findings by other investigators (3,4,5) and the suggestion is made that vagal inhibition is more readily responsive to nor-adrenaline since the bradycardia is abolished by atropine(2). Also, Lands and Howard say(6) nor-adrenaline is twice as effective as adrenaline in increasing the rate and amplitude of the perfused rabbit heart. On the other hand, Wakim and Essex(7) in a rather thorough comparison of adrenaline and nor-adrenaline effects in the dog maintain the cardiovascular responses to either agent are indistinguishable from one another. And a later study of isolated perfused hearts of rabbits, guinea pigs and dogs shows identical effects of adrenaline and nor-adrenaline on circulation through the heart with marked acceleration in all cases(8).

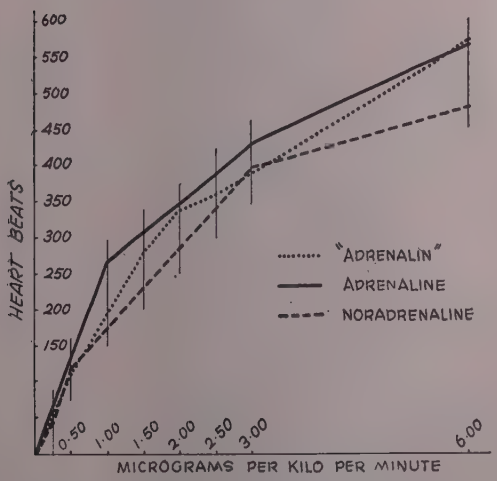

FIG. 1. Avg increase in heart beats over 10 min. period (5 min. injection and 5 min. recovery) at various dose levels of "adrenalin," adrenaline and nor-adrenaline.

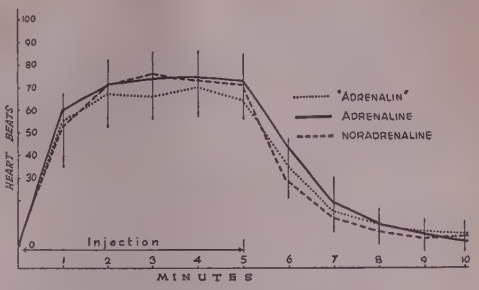

FIG. 2. Avg change in heart rate for each min. over 10 min. period at dose level of 3.00 μg per kilo per min. for 5 min. for "adrenalin," adrenaline and nor-adrenaline.

The results obtained here are in agreement with the above and with the views of others (9) who find that adrenaline and nor-adrenaline affect the excitability of the heart in a quantitatively and qualitatively similar manner.

Summary. No difference can be seen in the quantitative responses of the acutely surgically denervated heart of the cat to "Adrenalin," adrenaline or nor-adrenaline.

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Serologic Studies of Theiler's Mouse Encephalomyelitis Virus. (22487)

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Identification of TO(1) strains of mouse encephalomyelitis has generally been based on the signs of disease in mice and on histopathologic evidence, since neutralization tests in mice injected by the intracerebral route have been unsatisfactory. Theiler(2,3), by cross immunity tests, demonstrated an immunologic relationship between his TO viruses and the higher titered GD VII and FA strains. Strain variation among the TO viruses is suggested by differences in localization of paralysis in mice(4) and in their adaptation to growth in eggs and tissue culture (5).

This report presents a summary of neutralization tests using Theiler-free suckling mice, susceptible to infection by peripheral routes, and hyperimmune hamster sera produced with different Theiler strains. They demonstrate the group relationship and, in addition, serologic differences.

Methods and materials. Virus strains. The TO strains Nos. 4727, 4771, and 47218 were isolated in this laboratory from the intestines of albino mice(4). Strain No. 52102 was received from the American Type Culture Collection as a representative strain of TO virus. Its relationship to our strain No. 4727 has been shown(5) by tests in which mice injected with No. 4727 egg or tissue culture virus resisted challenge with No. 52102. GD VII Strain No. 51200 was received from Dr. Peter Olitsky. Virus stocks were stored in the dry ice chest as 20% suspensions of mouse brain and cords, or of chick embryo legs and wings. *Antisera* to strains Nos. 4727 and 52102 were prepared in hamsters by intraperitoneal injection of egg passage virus. An initial dose of 0.5 ml and 2 subsequent doses of 1 ml were given at from 3- to 4-day intervals. For certain lots of sera a preliminary intracerebral dose was given; to others a booster dose was given one month after third injection. Animals were bled 10-18 days after final dose. Antiserum to GD VII virus was prepared by Dean using strain received from Dr. Gilbert Dalldorf. Hamsters were immunized with 6 graduated doses of 10% suspension of infected mouse brains (0.125 to 1.0 ml) given over a period of 3 weeks. *In neutralization tests* a constant virus dose was combined with 5-fold serial dilutions of serum. Dilutions were made in 0.85% saline solution containing 10% beef infusion broth. Mixtures of virus and serum were held one hour at room temperature before injecting intraperitoneally in 0.05-ml doses into suckling mice from a Theiler-free colony. Usually the mice were from 3-6 days old; occasionally it was necessary to include those slightly younger or older. Viruses Nos. 4727 and 52102 were tested both as suspension of mouse brain and cord and of chick embryo legs and wings. The usual dosage represented approximately 100 mouse ID_{50}. Other amounts were also used. The effect on the serum titer is shown in the tables. Animals were observed daily for paralysis for a period of three weeks. Neutralization was considered definite if 60%, partial if 45-60% of the mice were protected.

Results. Until recently, because of the low peripheral infectivity of the Theiler viruses for standard laboratory mice, and the poor results obtained by intracerebral neutralization tests, identification of Theiler (TO) viruses by serologic methods has been impracticable and has depended on histopathology and signs of disease in mice. The complement-fixation test has been used for the GD VII and FA strains(6,7) and the hemagglutination test for GD VII(8). The work of von Magnus (9,10), who developed a Theiler-free mouse colony and established that such mice were susceptible to infection with TO virus by several peripheral routes, permitted a new approach to a serologic investigation of these low titered viruses. She used Theiler-free mice to evaluate hyperimmune cotton rat sera prepared with a Theiler TO strain and also presented data from other tests carried out by

TABLE I. Neutralization of Theiler Viruses by Homologous and Heterologous Hyperimmune Hamster Sera.

Sera		Virus suspension*							
		No. 4727			No. 4771	No. 47218	No. 52102		GD VII No. 51200
Strain	Dilution	E	E(x)	M	M	M	M	E	M
TO	5	3	3	3	3	3	3	3	3
No. 4727	25	3	3	3	3	2	3	3	2
	125	3	3	2	3	0	1	3	3
	625	2	0	0	1	0	0	0	0
	3125	0	—	—	—	—	0	—	—
TO	5	3	1	3	3	3	3	3	1
No. 52102	25	3	0	1	3	2	3	3	1
	125	1	0	0	2	0	3	3	1
	625	0	0	0	0	0	2	2	0
	3125	—	—	—	—	—	0	0	
GD VII	5	3	1	3	3	3	3	3	3
No. 51200	25	3	3	3	3	1	3	3	3
	125	3	0	3	3	0	1	1	3
	625	3	0	0	0	0	0	0	1
	3125	0	—	—	—	—	—	0	2

* Suspension used in dilutions which represented approximately 100 mouse ID_{50} except No. 4727 E(x). This suspension = 1000 mouse ID_{50}.

* With approximately 1/10 the dose of virus, this serum protected at a dilution of 15,625.

E = Egg passage virus, suspension of embryo legs and wings. M = Mouse passage virus, suspension of brains and cords.

3 = 60-100% infected mice survived for 21 days.
2 = 45- 60% *Idem*
1 = 30- 45% "
0 = 0- 30% "
— = Not done.

the intracerebral technic, stressing the superiority of the peripheral route for neutralization tests with neurotropic viruses. Dean(11), in this laboratory, also developed a colony of Theiler-free mice and showed that by whatever route tested they were more susceptible than latently infected animals to infection with the TO strain. They were also more susceptible to GD VII and FA viruses.

The availability of Theiler-free suckling mice permitted us to evaluate our antisera by the intraperitoneal neutralization technic. Table I incorporates the results of tests with three immune sera and five virus strains. In other experiments these strains were shown not to be neutralized by antisera for MM virus, Type II poliovirus strain Y-SK, or Coxsackie Group A, Type 1. None of the Theiler antisera had any protective effect against MM virus. Serum from control uninoculated hamsters had no neutralizing activity. The relationship between serum titer and the dosage of virus is indicated in Table I where 2 dosage levels of No. 4727 egg-passage virus are shown; and in Table II in which the results with 3 antisera on 2 different doses of strain No. 4771 have been summarized.

The TO strains isolated in this laboratory, the ATCC strain, and GD VII virus gave reciprocal cross reactions. However, the titer of each serum was higher for its homologous strain than for any other. This specificity is particularly noticeable in comparing the antisera for No. 52102 and GD VII. The latter had a high titer against its homologous strain and considerable neutralizing activity against all the strains. Antiserum to No. 52102 was

TABLE II. Influence of Virus Dose on Titer of Antisera.

Serum dilution	Antisera					
	No. 4727		No. 52102		GD VII No. 51200	
	100	1000*	100	1000	100	1000
5	3	3	3	3	3	3
25	3	3	3	2	3	3
125	3	1	2	0	3	1
625	1	0	0	0	0	0

* 100 and 1000 = No. of mouse ID_{50} of virus No. 4771, suspension of mouse brains and cords.

in general less reactive; but against virus No. 52102 it was definitely more active than was the GD VII antiserum.

All the antisera had a lower titer against strain No. 47218. This strain differs from the others in that it did not adapt to egg or tissue culture while strains Nos. 4727, 4771, and 52102 readily did so.

Summary. Satisfactory antisera to various Theiler strains were prepared in hamsters. Using Theiler-free suckling mice in neutralization tests, reciprocal cross reactions between GD VII and TO strains were demonstrated. Serologic differences within the TO group were also noted, the titer of a serum being in each case higher for the homologous than the heterologous strains.

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Occurrence of Melanocyte Stimulating Hormone (MSH) in a Transplantable Pituitary Tumor. (22488)

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Transplantable adrenocorticotropin (ACTH) secreting tumors have been produced in LAF_1 mice by Furth *et al.*(1,2,3) by ionizing irradiation. There was some indication that they released ACTH. To date, there has been no report on the ACTH content of the tumors. In the case of thyroid stimulating hormone (TSH) secreting tumors (3) an extremely high content of TSH has been reported. In view of many reports on the relationship of melanocyte stimulating hormone (MSH) and ACTH, it was of interest to assay the ACTH tumors for MSH, ACTH and vasopressin. It was found that the concentration of MSH was extremely high while the ACTH and vasopressin were relatively low.

Methods. Tumor Transplants. Several LAF_1 mice bearing Strain 2 adrenotropic tumor were sacrificed, the tumors were removed aseptically and homogenized with 5 volumes of isotonic saline. One-tenth ml of this suspension was injected intramuscularly into male LAF_1 mice weighing 22 to 28 g. Three weeks after implantation they were bilaterally adrenalectomized and injected subcutaneously with one mg each of desoxycorticosterone acetate (DOCA). The tumors were palpable about 8 weeks following implantation. Once palpable, they reached the size of 1-6 g within a few weeks. All the reported experiments were carried out with tumors which had been transplanted twice following receipt in our laboratory. *Assays.* ACTH assays were conducted by the intravenous technic described by Munson, Barry and Koch(4). Vasopressin determinations were by the standard U.S.P. XV method. All samples for ACTH and vasopressin were prepared by homogenization with 0.1N hydrochloric acid followed by dilution with isotonic saline. MSH determinations were made using the *in vitro* method of Shizume and Lerner(5). Samples were extracted with 10

TABLE I. MSH, ACTH and Vasopressin Content of 12 Adrenotropic Tumors.

Tumor wt (g)	MSH (u/g)	ACTH (USP u/mg)	Vasopressin (I.U./mg)
3.05	1.0×10^7	.0016	<.0005
4.96	7.5×10^6	<.0005	"
1.60	2.1 "		
.28	4.5 "		
2.75	3.5 "		
5.05	1.0×10^7	.0003	<.0015
2.15	1.5×10^6		
.40	1.0×10^7		
.97	1.6 "	<.0005	"
1.98	5.7×10^6		
.41	3.3 "		
.30	5.7 "		
Mean and S.E.	$6.7 \pm 1.3 \times 10^6$		

volumes of approximately 0.1N acetic acid and diluted with frog Ringer solution. The final pH was approximately that of the diluting solution. An MSH preparation (CDF-G456) containing 4.0 x 10^9 units/g was used as a standard.

Results. Table I summarizes the assay data on 12 adrenotropic tumors removed from LAF_1 mice after 2 transplantations. All were transplanted and sacrificed at the same interval. The MSH concentration, expressed in terms of fresh tissue equivalents, was 6.7 $\pm$ 1.3 x 10^6 units/g which was approximately that found in normal mouse pituitaries (Table II). On the contrary, the ACTH concentration was less than 1/20 that of the normal pituitary. Vasopressin determinations confirmed that there was no posterior pituitary contamination. Histological structure of the tumors and pituitaries of adrenalectomized mice indicated that the tumors were of anterior lobe origin and not from the intermediate or posterior lobes.

Samples of skeletal muscle from the hind leg not implanted with tumor cells and from adrenalectomized mice were also assayed. Both contained less than 1/1000th the concentration of MSH found in the tumors. There was a suggestion that the concentration in the muscle of tumor bearing animals was higher than the normals but both were so low that the results were of doubtful significance.

Discussion. The melanocyte stimulating hormone, sometimes referred to as intermedin, was shown by Zondek and Krohn(6) to be derived from the intermediate lobe of the pituitary. Many workers have pointed out that MSH and ACTH possess many of the same chemical and physical properties but are distinct substances. Lerner and Lee(7) isolated swine MSH which possessed very little if any ACTH activity. However, pure swine ACTH has about 1% inherent MSH activity(8). Shizume and Lerner(9) have shown that blood and urinary MSH levels can be lowered by cortisone and hydrocortisone. The above observations indicate that there may be a common pathway for the synthesis of ACTH and MSH. Judging from the data of Furth *et al.*(3) on adrenal responses it appears that the original tumors contained more ACTH than that reported in this study. It is well known that tumors tend to lose their functional identity as a result of repeated transplantation. In this case, the capacity for producing ACTH must be less than for MSH.

Of particular interest was the MSH content of the tumors as compared to that of the normal mouse pituitary. Within limits of error of the assay, the concentrations were the same yet the tumors were in some cases 2 to 3,000 times the size of the pituitary. If the tumors were releasing the hormone at the same rate as the pituitary, the blood and urine values for MSH should be markedly elevated. Therefore, the LAF_1 mouse with the Strain 2 adrenotropic tumor should be an excellent animal to study the physiology and pharmacology of MSH in the mammal.

Summary. Strain 2 adrenotropic tumors of LAF_1 mice were assayed for MSH, ACTH and vasopressin. The concentration of MSH was approximately that of the normal mouse pituitary. ACTH and vasopressin concentrations were very low. The possible relation-

TABLE II. MSH and ACTH Content of Pooled Mouse Pituitaries.

	No. animals	MSH (u/g)	ACTH (USP u/mg)
Tumor bearing	11	9.0×10^6	.022
Normal	6	7.0 "	.043
Adrenalectomized	6	$1\text{-}2 \times 10^7$	.039

ship of MSH and ACTH was discussed. The adrenotropic tumor-bearing animal should be advantageous in the study of the action of MSH in the mammal.

The authors are indebted to Dr. Jacob Furth for supplying the tumor-bearing mice for transplantation and to Dr. Aaron B. Lerner for a standard preparation of MSH.

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Effect of Magnesium Ion on Glycogen Fraction Synthesis in Rat Tissues.* (22489)

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Magnesium is intimately associated with carbohydrate metabolism through its function as an activator in enzymatic processes involving phosphatase(1). However, opposing views exist regarding the promotion of glycogen synthesis by magnesium. Injection of Mg salts was claimed by Franke(2) to promote hepatic glycogenesis, but according to Peters and Van Slyke(3) the data have not been confirmed and are not convincing. Reid(4) on the other hand demonstrated a 6-fold increase in liver glycogen of cats after injecting Mg as chloride or gluconate. No reference to the effect of Mg on the synthesis of the glycogen fractions of Bloom(5) has been found by the writers.

This report presents the results of glycogen fraction studies in heart, liver and skeletal muscle of the rat in an attempt to elucidate the relationship of the TCA soluble and TCA insoluble glycogen fractions after Mg salt injections.

Methods. White rats weighing between 200 and 400 g were fasted 18 hours prior to injection I.P. with 0.2M Mg sulfate. Each rat received approximately 1.6 mg of Mg ion per 100 g of body weight. The sexes were equally mixed. Control rats were injected with equivalent molar concentrations of sodium chloride. The animals were then sacrificed by decapitation at one or 4 hours. The entire heart and samples of liver and gastrocnemius muscle were taken for analysis for TCA soluble and TCA insoluble glycogen fractions using the procedure outlined by Bloom, *et al.*(5). After hydrolysis of the glycogen in sulfuric acid, the isolated glycogen samples were measured quantitatively by the anthrone procedure of Seifter, Dayton, Novic and Muntwyler(6). A separate series of 12 fasted rats was injected with Mg sulfate at the same dose level as the rats in the glycogen series for the serum Mg data. These were sacrificed after one hour. Another 15 rats similarly treated were sacrificed after 4 hours. An additional 12 untreated rats were used as controls. Blood was withdrawn by direct heart stab and analyzed for serum Mg using the method of Orange and Rhein(7) as modified by Platner and Hosko(8).

The results of the glycogen and serum Mg determinations are presented in Table I.

* Supported by grants from Lederle Laboratories and Department of Health, Education and Welfare.

TABLE I. Glycogen Content (mg % ± S.E.) of Rat Tissues as Affected by Magnesium Ion.

	Control	Magnesium inj. 1 hr	4 hr
Heart			
TCA insoluble glycogen	110± 9.7 (19)*	131± 7.3 (15)	155±11.6† (15)
TCA soluble glycogen	187±25.5 (19)	250± 28.3 (15)	219±21.9 (15)
Total glycogen	297±31.8 (19)	381± 29.6 (15)	374±22.3 (15)
Muscle			
TCA insoluble glycogen	107±10.1 (19)	137± 14.9 (15)	184±13.3† (15)
TCA soluble glycogen	167±16.7 (19)	167± 12.7 (13)	174±24.4 (15)
Total glycogen	274±18.7 (19)	312± 22.9 (13)	358±27.4† (15)
Liver			
TCA insoluble glycogen	164±17.5 (19)	224± 13.7† (14)	196±20.7 (30)
TCA soluble glycogen	382±70.7 (17)	796±144.8† (15)	402±58.9 (28)
Total glycogen	536±75.0 (18)	958±150.9† (14)	620±75.4 (27)
Serum magnesium	1.73±.08 (12)	5.21±.25 (12)	1.45±.09 (15)

* No. in parenthesis refers to No. of animals used.

† Significant to 98% level.

Discussion. One hour after Mg sulfate injection there was a sharp rise in total liver glycogen. Most of this rise is due to the TCA soluble fraction, although the increase in TCA insoluble fraction is also significant, ($P = 0.02$). Four hours following the Mg injection there was a significant increase in the TCA insoluble fraction in heart tissue. Skeletal muscle also showed at this time a significant increase in both the TCA soluble and TCA insoluble fractions. The data suggest that the liver reflects changes in blood Mg levels much more readily than does skeletal muscle or heart tissue. The serum Mg levels support this finding in that the highest serum Mg levels coincide in time with the greatest rise in liver glycogen values.

The heart tissue shows the next greatest rise and skeletal muscle appears to be least affected after one hour, but both tissues show significant increases after 4 hours. The delayed response of the heart and especially skeletal muscle may be due simply to the slow rate of entrance of the Mg ion into muscular tissue. Smith, *et al.*(9) showed that Mg distributes itself in the body more like Na than K and that complete distribution requires more than 4 hours to enter spaces other than extracellular. Delayed entrance of the Mg ion into muscular tissue may also be due to dilution by body fluids. After 4 hours, as the serum Mg falls to control levels, the total glycogen also declines in heart and liver but continues to rise slightly in skeletal muscle.

Although both glycogen fractions follow a similar pattern, the TCA soluble fraction appears to be synthesized at a slightly greater rate than the TCA insoluble fraction. The data imply that the Mg ion *in vivo* promotes synthesis of both glycogen fractions in the 3 tissues studied. This finding is quite significant since very few substances have been reported which increase the synthesis of the TCA insoluble fraction. The sharp increase of the soluble fraction in liver one hour following Mg injection implies a preferential effect of this ion on the synthesis of this fraction in liver pointing up the difference in metabolic pattern between this tissue and skeletal muscle.

Summary. Magnesium sulfate injected I.P. into rats promotes synthesis of both TCA soluble and TCA insoluble glycogen fractions in the liver after one hour. Heart and skeletal muscle show a significant rise in TCA insoluble glycogen fraction after 4 hours. Skeletal muscle also shows a significant rise in total glycogen after 4 hours. Magnesium appears to be one of the few substances which promote synthesis of the TCA insoluble glycogen fraction.

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Potentiated Inhibition of *Escherichia coli* by Certain Combinations of Agents. (22490)

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Much useful information has been obtained on the modes of action of chemotherapeutic agents, including tumor-inhibiting agents, by inhibition analysis in bacterial systems. In a number of cases the mode of action appears to be the same in bacterial and mammalian systems. Therefore, it is not unreasonable to test agents or combinations of agents against bacteria to serve as a guide for tests in mammals. In this laboratory, candidate anticancer agents are tested singly and in combinations as growth inhibitors for bacteria prior to testing against neoplasms in animals. This report presents some of the data that have been obtained for combinations of agents in tests with bacteria.

Methods. To screen for potentiating growth-inhibitory activity, a simple test method was devised using *Escherichia coli* (ATCC No. 9637) as the test organism. The use of this organism grown in a simple, chemically defined glucose-salts medium is ideal for biochemical studies of this kind because the observed action of specific inhibitors or antimetabolites is not obscured by the presence in the medium of the chemically complex metabolites required in similar studies on more fastidious organisms. Duplicate paper discs saturated with known concentrations of a candidate compound were placed on the surfaces of minimal agar cultures of *E. coli* in a control plate and in a test plate. The control plate contained no test compound, whereas the test plate contained a subinhibitory concentration of the second member of the pair of candidate compounds being tested for potentiation. If the diameter of the zone of inhibition around the paper disc on the test plate was greater than 2 times that on the control plate, then the combination was considered worthy of further study. Of approximately one thousand pairs of compounds tested, approximately one hundred pairs gave evidence of significant additive or potentiated inhibition. Although the agar-plate method described above was quite useful for rapidly screening combinations for additive activities, it was considered desirable to subject the combinations that showed the more promising activities to another test, which would permit a quantitative evaluation of the combinations. For this purpose, the method of Elion, Singer, and Hitchings(1) was used, and *E. coli* (ATCC No. 9637) was used instead of *Lactobacillus casei* or *Streptococcus faecalis*. The minimal medium of Davis and Mingioli(2) was used with the omission of the agar. A 24-hour culture of *E. coli* was adjusted by addition of sterile water to have a transmission of 55 $\pm$ 2% (Bausch and Lomb Monochromatic Colorimeter with a 660 mμ interference filter), and then diluted 1:10 with sterile water. Each 10-ml tube of medium was inoculated with 0.1 ml of this suspension. The extent of growth was determined turbidimetrically after incubation at 37° for 16-18 hours. The effects of the 2 compounds being tested upon the growth of the bacteria were determined by testing various levels of the compounds alone and in combination. In each case, the quantity of the compound required to cause half-maximal inhibition was determined graphically as shown in Fig. 1 and 2. The fractional inhibitory concentra-

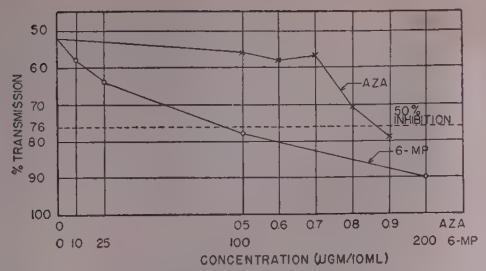

FIG. 1. Inhibition of growth of *E. coli* by azaserine and by 6-mercaptopurine.

TABLE I. Concentrations of Azaserine and 6-Mercaptopurine Causing Half-Maximal Inhibition of Growth of *Escherichia coli*.

Azaserine		6-Mercaptopurine	
μg/10 ml	Fractional inhibitory conc.	μg/10 ml	Fractional inhibitory conc.
.00	.00	88.8	1.00
.10	.12	8.7	.10
.15	.18	7.3	.08
.20	.23	4.4	.05
.25	.29	3.4	.04
.30	.35	3.0	.03
.86	1.00	0.0	.00

tion for each compound in a particular combination was calculated by dividing the concentration of the inhibitor present in the combination by the quantity of inhibitor which would be required to give half-maximal inhibition by itself. (Table I). By plotting the fractional inhibitory concentrations as shown in Fig. 3, it is possible to determine the effectiveness of the combination. If the 2 compounds have effects which are additive, the points will fall on a straight line connecting unity on the ordinate with unity on the abscissa. Deviations to the left of this theoretical straight line indicate potentiation; deviations to the right might represent interference or antagonism between the drugs. By drawing intersecting straight lines through the experimental points, a point is obtained where the combined fractional inhibitory concentrations reach a minimum. This point represents the point of maximal effectiveness. The numerical sum of the coordinates of this point can be used to indicate the degree of effectiveness of the combination—the smaller the value of the sum, the more effective the combination. Therefore, by comparing the magnitudes of these sums, it is possible to compare the activities of various combinations. Sums that were determined from data obtained in these experiments are presented in Table II. Each experiment was performed in duplicate or triplicate, and each sum listed in Table II is the mean value for these experiments. To permit correlation of antibacterial activity and anticancer activity, the results of some tests with Sarcoma 180, Adenocarcinoma 755, and Leukemia L1210 are indicated in Table II. Some of the combinations are listed twice in the table to facilitate comparison with the values for other combinations.

Comments. The usefulness and limitations of this type of experiment have been discussed (1), and the chief purpose of the present paper is to present the data for combinations that have been tested in this laboratory. Although these data could be used in discussions of biochemical mechanisms, such discussions will not be presented here.

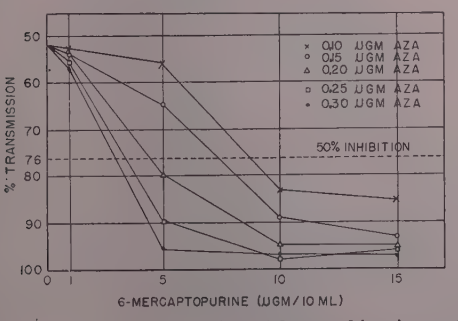

FIG. 2. Inhibition of *E. coli* by combinations of azaserine and 6-mercaptopurine.

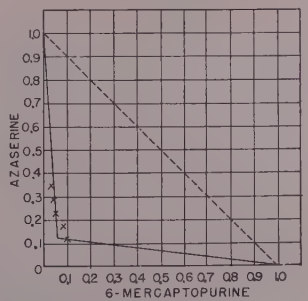

FIG. 3. Effects of combinations of azaserine and 6-mercaptopurine upon growth of *E. coli* in terms of fractional inhibitory concentrations.

Several combinations were found to be outstandingly potentiating. Perhaps the combinations of Daraprim with canavanine sulfate and of 3-(1,2,4-triazolyl)alanine with canavanine sulfate were the most active, since the values presented in Table II for these combinations were based upon subinhibitory levels of canavanine sulfate and the true values would therefore be lower than the tabulated values.

TABLE II. Effectiveness of Combinations of Drugs.

Combination	Sum of fractional inhibitory conc. of most effective combination for *E. coli*	Potentiation of anticancer activity* Positive	Negative
Sulfanilamide + Desoxypyridoxine	.24		L1210†
+ Adenine sulfate	.39	Not tested	
+ Daraprim‡	.55		L1210† Sa 180†
+ 6-Mercaptopurine	.57	L1210† Sa 180† (?)	
+ Hypoxanthine§	.58		L1210† Sa 180†
+ DL-Ethionine	.59	L1210†	Sa 180†
+ 3-(1,2,4-Triazolyl)alanine	.67		L1210†
Azaserine‖ + 6-Mercaptopurine	.30	L1210 Sa 180	
+ 3-(1,2,4-Triazolyl)alanine	.47		L1210
+ 2,6-Diaminopurine	.67	Not tested	
+ Desoxypyridoxine	.76	Ad 755 Sa 180	L1210
Daraprim + Canavanine sulfate§	.13		L1210
+ 2,6-Diaminopurine	.47	Not tested	
+ Sulfanilamide	.55		L1210 Sa 180
Desoxypyridoxine + 3-(1,2,4-Triazolyl)alanine	.13		Sa 180
+ DL-Ethionine	.23		Sa 180
+ Sulfanilamide	.24		L1210
+ Benzoic acid hydrazide	.32	Sa 180	
+ Isonicotinic acid hydrazide	.43	"	
+ p-Aminobenzoic acid hydrazide	.52	"	
+ 1, 5-Diaminobiuret	.67	"	
+ Azaserine	.76	Ad 755 Sa 180	L1210
+ Methionine sulfoximine	1.21	Not tested	
3-(1,2,4-Triazolyl)alanine + 6-Mercaptopurine	.09		L1210
+ Canavanine sulfate§	.11		L1210
+ Desoxypyridoxine	.13		Sa 180
+ Adenine sulfate	.14	Not tested	
+ 6-Chloropurine	.15	"	
+ 2, 6-Diaminopurine	.15	"	
+ 5-Bromouracil	.44	"	
+ Azaserine	.47		L1210
+ 2-Thiazolealanine	.60	Not tested	
+ Sulfanilamide	.67	"	
Streptomycin sulfate + 6-Mercaptopurine	.63	"	
+ Dithiouracil	.79	"	
+ Azaserine	.84	"	

* The experimental neoplasms used were Leukemia L1210, Sarcoma 180, and Adenocarcinoma 755.

† In tests of anticancer activity, A-methopterin was used instead of sulfanilamide.

‡ 2, 4-Diamino-5-p-chlorophenyl-6-ethylpyrimidine.

§ Calculations were based upon a subinhibitory level of this compound, and therefore, the combination is perhaps much more active than figure in table indicates.

‖ O-Diazoacetyl-L-serine.

It is interesting to note that the combinations that were most effective against *E. coli* were often ineffective in the tests against neoplasms, whereas some combinations that were less effective against *E. coli* were effective against these neoplasms. For example, although the value of the sum of the fractional inhibitory concentrations for azaserine plus 6-mercaptopurine is 0.30 and that for 3-(1,2,4-triazolyl)alanine plus 6-mercaptopurine is 0.09, the former combination was effective against leukemia L1210 whereas the latter was ineffective. Other similar examples could be picked from the table. On the other hand, combinations that have been found to be potentiating in tests with animal neoplasms are also potentiating in inhibiting the growth of *E. coli*. Although the correlation between the data for *E. coli* and the indicated neoplasms is not as good as would be desired, the bacterial screen does indicate combinations that should be tested against neoplasms in animals. For example, the potentiating effects of combinations of desoxypyridoxine and acid hydrazides were first detected in tests with bacteria.

It is also possible that some of the combinations that are potentiating against *E. coli* might be of value in studies related to the chemotherapy of infectious diseases caused by bacteria or protozoa.

Summary. Data are presented for the potentiating effect of several combinations of compounds in inhibiting the growth of *E. coli*. Data of this kind can serve as guides for the selection of combinations of agents to be tested in the chemotherapy of cancer and of infectious diseases.

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Tremor Production in Cats Given Chlorpromazine. (22491)

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The production of a Parkinson-like state in humans receiving large daily doses of chlorpromazine has been observed clinically by us and others(1-4). To study further this phenomenon, chlorpromazine in different dosages and for variable periods of time, was injected in cats and the results observed. It was anticipated that a state of rigidity would be most prominent if any effects were obtained, but an unexpected feature was the appearance of an alternating tremor at rest. This has previously been produced in primates by fulgurations in the region of the subthalamus(5,6), ablations of the cerebellar cortex and nuclei (7), fulgurations in the mesencephalic and pontine tegmentum(8), electrical stimulation of the medial reticular formation(9), and by use of Reserpine(10). Folkerts and Spiegel (11) observed an alternating tremor during electrical stimulation of the midbrain tegmentum in the cat.

Methods. Twenty-eight adult cats were observed prior to and following the intramuscular injection of chlorpromazine to determine the quality of placing and hopping reactions, labyrinthine righting reactions, stütz response, both dependent and supine, pupillary reaction, general performance, gait, tendon reflex activity, muscle tonus, and tremor. Drug doses were calculated on the basis of those frequently employed for a 70 kg human which range from 300 to 2500 mg/day and were proportioned according to the cat's weight. Doses were given once daily. They varied in amount as mentioned and were administered as single injections, intermittent injections, or consecutive daily injections for as long as 30 days. Tremors and other drug

effects were recorded by motion picture camera and by this means tremor frequency was analyzed.

General observations. Tremor. Of the 28 animals studied, 13 (46%) developed a tremor of one or more extremities or of the head during some phase of the investigation. The basic response consisted of a coarse, alternating tremor at rest with a frequency of 6-8/sec. as judged by observation and motion pictures. Some common variations of this basic response included: (1) more rapid tremor of smaller amplitude in which the rate was estimated at 16-18/sec. by observation; (2) slower tremor of greater amplitude at about 4-6/sec.; (3) lack of spontaneously occurring tremor, but one which could be precipitated by a light tap on one of the footpads, affecting either the stimulated limb or another extremity without regard to laterality; (4) irregular tremor which varied in frequency or amplitude. In no instance was an intention tremor noted. Tremor production was dependent upon having the animal in a supine position (either in a wooden trough or supported on a table top) with a maximum degree of relaxation. In the 13 animals exhibiting a tremor, this disappeared when medication was stopped, and frequently reappeared upon readministration, on at least one occasion in all animals, and up to 6 times in certain ones during the period of observation. Tremor production bore only a partial relationship to dosage of the drug, in that doses below 500 mg human equivalents and above 2500 mg human equivalents were ineffective; amounts between these limits were effective at various times in different animals.

Other drug effects. Among the drug effects noted in this group were variable degrees of shivering, diarrhea, relaxation of the anal sphincter, lethargy, and frequent loss or diminution of the various postural and righting reflexes referred to earlier. However, there was no obvious change in the stütz responses, tendon reflexes, gait (except for infrequent impairment of motor function in the injected limb), or of pupil size and light response.

Absence of tremor. In the 15 cats which did not develop a tremor there was more pronounced lethargy and less response to stimulation than in the tremor animals. However, righting reflexes and postural responses remained relatively normal. Shivering, diarrhea, anal sphincter tone, gait, pupillary size and light response, and tendon reflexes were not noticeably different from those in the group with tremors.

Secondary observations. In 2 animals of the tremor group spontaneous movements which might be described as "swimming movements" were occasionally observed. In some instances these occurred in association with the tremor. These movements took the form of rhythmical alternating flexion-extension of the forelimbs accompanied by abduction of the toes and extension of the claws in the extended limb, and adduction of the toes and withdrawal of the claws in the flexed limb. When tremor accompanied this phenomenon, it was present in either position of the limbs, but exhibited greater amplitude and excursion in the flexing limb. In one animal it was present in the lateral, as well as the supine position. In four of the cats with tremor a stimulus such as a light tap on the footpad of a hind limb produced extension or flexion of a forelimb without regard to laterality. In all such instances the responses followed an ascending course, as forelimb stimulation did not elicit a hind limb response.

In another instance light constant tapping of a tendon at first produced no observable muscle contraction, but after several such stimuli a phasic tendon reflex appeared and increased progressively in amplitude of excursion for the next four to five taps, then showing a gradual decline until it could no longer be elicited. After a 60 second interval, the same phenomenon could be reproduced under similar conditions. A fourth effect occurring in varying degrees in four of the animals and independent of tremor production, was a lack of response to nociceptive stimuli, elicited by pinching or applying heavy pressure to the tail. The animals would either appear to be totally oblivious to the stimulus or would merely respond by a flick of the tail without turning the head in the direction of

the stimulus. This was in marked contrast to the behavioral responses of the controls.

Effects of prolonged administration. In 2 cats which received large continued doses of the drug (1500 and 2000 mg human equivalents) for 30 days, rigidity was observed in all 4 extremities and the trunk without any significant change in postural reflexes, righting reactions, and tendon reflexes. Upon cessation of medication the rigidity disappeared within ten days and the animals appeared to have returned to their normal state. In 2 other cats which were initially injected for 7 consecutive days with 300 and 400 mg human equivalents respectively, after which injections were discontinued for 14 days and then resumed in a like amount for 7 more days, no changes were observed as regards rigidity, tendon reflexes, righting reactions or postural reflexes.

Discussion. The tremor which we have observed with its variability in time, amplitude, regularity and anatomical location, but with a basic 6-8/sec. rhythm and appearing at rest, closely resembles that produced by the stimulation(11) and destruction(8,9) experiments of other workers, both in the same species and higher vertebrates, and that produced in man by the use of chlorpromazine(1-4). These earlier anatomical and physiological studies seemed to implicate a portion or portions of the reticular formation as a source for this tremor. More recent pharmacological studies (12,13), utilizing cholinergic, anticholinergic and ataraxic drugs which produce electrical changes in this region, appear to give additional support to the concept that activation of this anatomico-physiological system is in some way implicated in the appearance of a tremor at rest.

The dosage of chlorpromazine employed by us to produce tremor was in most instances comparable to that reported by Himwich and Rinaldi(12,13) as being within the range which produced a low voltage, fast record (activation) of the motor and limbic cortices, caudate nuclei, and the medial thalamic nuclei. We have noted that in the animals which did not develop tremor as compared to those which did there was reduced motor activity, drowsiness, and diminished interest in the immediate surroundings.

This behavioral observation links changes in electrical activity of the reticular formation induced by chlorpromazine, the state of alertness as dependent on the activity of this system(14) and the presence of a tremor at rest. We do not wish to imply that the reticular formation is necessarily the source of this tremor, but that it may merely reflect activity originating at more cephalic or caudal levels which traverses this region.

The appearance of parkinsonism with tremor in humans which may result from the use of chlorpromazine shows wide variation in time of appearance after institution of drug therapy, but in all cases has been reversible after discontinuance of the drug. In contrast to human observations the cats which react acquire a tremor after single injection; this difference may represent a species variation.

Since it is possible that sensitization to the drug may be a factor in the production of a tremor, the finding that initial doses sometimes produced tremor whereas interrupted doses did not, was felt to present some evidence that such a factor was not operative. Another observation of interest was the similarity of the "swimming movements" to those obtained by stimulation within the midbrain, which were termed tegmental responses by Ingram, *et al.*(15); however, it is possible that they might represent pleasure or nursing movements. The responses obtained by tactile stimulation and by repetitive tapping (of extensor tendons), were not unlike the irradiation and recruitment responses which have been described for spinal reflexes.

Summary. In a series of 28 cats injected with varying doses of chlorpromazine, 13 (46%) developed tremors. The basic tremor was present at rest and disappeared on movement. The rhythm was most often 6-8/sec., but a few animals displayed a faster or slower rate. The tremor was reversible and reproducible. The implications of these observations relative to human parkinsonism are discussed.

The authors are indebted to Doctors Ingram, Sahs and Correll for valuable assistance and suggestions.

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Ejaculatory Response Induced by Potassium Chloride in Small Mammals. (22492)

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Artificial ejaculation in laboratory animals has been produced by electrical stimulation and by drug action, the latter consisting of a hypnotic followed by a stimulant. No clear description of the properties of the secretions obtained has been reported, nor has either method been described as capable of producing a complete ejaculation containing sperm as well as secretions from the accessory organs of reproduction. A high incidence of ejaculation occurs in the final convulsions of a dying animal. Loewe(1) has termed this phenomenon terminal ejaculation in contrast to intravital ejaculation which neither coincides with death nor is followed by death after a short interval of time. Potassium chloride has been found to produce an intravital ejaculation in mice following intraperitoneal injection of sub-lethal doses. This investigation was undertaken to determine the mechanism by which the ejaculations were induced and the nature of the secretions.

Methods. The animal species employed were the albino mouse, albino rat (Wistar), hamster, guinea pig, pigeon, and ground squirrel. All animals used were adult males. The ground squirrels were trapped by snaring in the vicinity of Lafayette, Ind., and were kept in captivity about 6 months prior to their use. Other animals were obtained from breeders. Chemical agents were administered by intraperitoneal injection into the lower quadrant of the abdomen. Ejaculation time was measured from the time of injection until the first appearance of seminal fluid at the penis. Weights of ejaculates were made by collection on weighed glass microscope slides. Fructose analyses were made by the colorimetric method of Roe as modified by Mann(2). Seminal vesicles were removed through a midline incision in the abdomen cephalad the base of the penis. Isolated seminal vesicles were used to test the *in vitro* action of chemical agents by the method described by Stone and Loew(3). A seminal vesicle, placed in a bath of oxygenated Locke's solution to which

* This work was carried on in partial fulfillment of requirements for degree of Ph.D.

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TABLE I. Ejaculatory Response of Different Animal Species to Intraperitoneal Injections of Potassium Chloride.

Species	No. tested	KCl dose range, mg/kg	No. ejac.	% ejac.	Effective dose range, mg/kg
Mouse	69	50–1000	41	68.3	100– 800
Rat	12	50– 600	10	83.3	100– 600
Hamster	11	25– 600	5	45.4	400– 600
Ground squirrel	6	175–1200	4	66.7	800–1200
Guinea pig	6	50– 150	4	66.7	100– 150
Pigeon	7	600	0	0	
Cat	1	300	0	0	

1.0% dextrose had been added, was attached to a lever writing on a slowly moving kymograph. Chemical test agents were added directly to the bath.

Results. Intraperitoneal injections of KCl in mice evoked a series of symptoms which were, in order of their appearance: 1) signs of discomfort, 2) 3 to 5 stretching movements with extension of the hind legs, and 3) arching of the back (opisthotonus). All symptoms occurred within a period of 30 seconds to one minute during which time the animal ejaculated. Often a coagulated plug followed the ejaculation. The most effective dosage of KCl for the mouse was 400 mg/kg although positive results were obtained by doses ranging from 100 to 800 mg/kg. Different dilutions of KCl were tried from 2.5 to 5.0%, the best effect being produced with 400 mg/kg of 3.5%. Other animal species showing positive results with KCl injections were rat, hamster, ground squirrel, and guinea pig (Table I). The cat and pigeon were negative. The ground squirrel required dosage from 800 to 1200 mg/kg to be effective but tolerated all doses. The hamster required 400 to 600 mg/kg KCl.

Epinephrine, nor-epinephrine, and acetylcholine also induced ejaculation in mice; however, ejaculation caused by acetylcholine more nearly resembled that produced by KCl than did that produced by epinephrine inasmuch as the ejaculate was more forceful, of larger quantity and more frequently followed by plugs. Drugs which block autonomic action such as atropine and Priscoline successfully abolished ejaculations normally caused by acetylcholine and epinephrine but did not block KCl. Deep ether anesthesia blocked KCl ejaculation while light ether anesthesia did not. Often ejaculation followed recovery from anesthesia by ether during which KCl had been given. Both acetylcholine and epinephrine stimulated excised seminal vesicles *in vitro* in much weaker concentrations than those inducing ejaculation *in vivo*. KCl even in massive doses did not act directly on the smooth muscle of the seminal vesicles. Chemical analyses of the ejaculates showed the presence of fructose but without correlation between ejaculate weight and fructose content. After removal of the seminal vesicles no ejaculatory responses were obtained with repeated tests up to 30 days. Upon postmortem examination, the animals showed complete absence of seminal vesicles but with normal appearing prostate gland. KCl-induced ejaculation exhibits an all-or-nothing response as any dose that is effective produced a maximal response.

Discussion. These experiments have demonstrated that artificial ejaculation can be induced in mice by the intraperitoneal injection of sublethal doses of potassium chloride with no apparent harm to the animals even on repeated daily injections. This response has also been obtained in other rodent laboratory and one wild species. Attempts to standardize quantitatively the ejaculatory response by measurement of ejaculation time or weight were not successful. Because the response was obtained only with compounds which contained the potassium ion the effect may possibly be attributed to the reported actions of this ion on nerves. Potassium ions alter the resting potential of nerve membrane(4) and injection of potassium into fluid perfusing ganglia causes a discharge in both pre- and

post-ganglionic fibers as well as liberation of acetylcholine(5). Acetylcholine or epinephrine injections also induced ejaculation in mice but pretreatment with atropine and Priscoline prevented, respectively, the ejaculations induced by these drugs but not those induced by KCl. Ejaculations induced by acetylcholine were qualitatively and quantitatively better than those induced by epinephrine, suggesting predominance of parasympathetic control of the efferent ejaculatory pathway.

It is assumed that KCl produces only ejaculation of the fluid present in the seminal vesicles because no sperm were ever found in the ejaculates. Repeated daily application of KCl apparently does not injure the animals so it is believed that it is a safe means of inducing ejaculation of seminal fluid without sacrificing the animals for collecting fluid for bio-assay studies.

Summary. Intraperitoneal injection of KCl has been demonstrated to induce intravital ejaculation in mice, rats, guinea pigs, hamsters and ground squirrels, but not in pigeons or cats. The response is an all-or-nothing response regardless of the size of the dose. A dose of 400 mg/kg of 3.5% KCl was found to be the most effective in mice. Only potassium-containing salts were effective in producing ejaculation. It was not possible to standardize the ejaculatory response by measuring ejaculatory time or by weighing the ejaculate. Extirpation of the seminal vesicles, bilateral castration, and analysis of the fructose content of the semen indicated the seminal vesicles as the source of the fluid. No sperm were found in the fluid at any time. Repeated daily injections of KCl cause ejaculation without apparent harm to the animals.

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Uterine Growth Stimulating and Testicular Growth Suppressing Activities of 17α-Ethinylandrostane-3β,17β-diol, Its Δ^5-Analog and Derivatives. (22493)

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Comparative studies of biological activity of 17α-ethinylandrostane-3β,17β-diol and its Δ^5-analog(1) have been concerned largely with androgenic and luteoid potency as related to other androstane and pregnane derivatives (2-5). Our observations have indicated that these steroids are characterized by predominantly estrogenic activity (growth promotion of infantile rat uterus and inducement of vaginal cornification in ovariectomized rats). The present report is a structure-activity relationship survey of ethinylandrostanediol, ethinyl-Δ^5-androstenediol, and esters of each, with respect to relative activity in stimulation of uterine growth (UGSt) and suppression of testicular growth (TGSp) in immature intact rats. Observations on the UGSt and TGSp activities of estradiol-17β, ethinylestradiol and Δ^5-androstenediol are included for reference purposes. Definitive endocrinological studies on certain compounds of the series will be reported separately.

Materials and methods. Immature male or female Sprague-Dawley rats weighing 35-40 g were used. Unless noted otherwise all test substances were dissolved in cottonseed oil containing 10% ethanol v/v and administered subcutaneously in 0.2 ml of vehicle. The uterine growth tests were performed essentially in accordance with procedures outlined by

TABLE I. Uterine Growth Stimulating and Testicular Growth Suppressing Activities of Estradiol-17β.

A. Uterine growth stimulating (UGSt) activity

Treatment	Dose, mg/kg/day	No. of rats	Uterine wt, mean ± S.E., mg	% stimulation	ED_{50}, mg/kg/day
None	—	8	38.4 ± 3.4	—	
Estradiol-17β	.00037	8	49.5 ± 3.5	15	
	.00075	8	77.8 ± 4.9	55	.0008
	.0015	8	89.8 ± 7.1	72	
	.003	8	96.8 ± 4.2	82	

B. Testicular growth suppression (TGSp) activity

Treatment	Dose, mg/kg/day	No. of rats	Testicular wt, mean ± S.E., mg	% suppression	ED_{50}, mg/kg/day
Controls					
Initial	—	10	220 ± 10	—	
Final	—	9	556 ± 17	—	
Estradiol-17β	.05	5	406 ± 36	45	.055
	.10	5	301 ± 17	76	
	.20	5	251 ± 12	91	

Lauson *et al.*(6). The test material was injected once a day for 3 days. On the 4th day, 24 hours after the last injection, the uteri were removed, blotted and weighed on a micro-torsion balance. For testicular growth suppressing activity the test agents were injected once a day for 5 days. On the 3rd day after the last injection the testes were removed, cleaned and weighed. At least 3 dose levels of each compound were employed within the active range. The dose of each compound which effected 50% of maximum change (ED_{50}) was calculated from log-dose probit graph paper according to the method of Miller and Tainter (7). A uterine weight of 110 mg was used as the maximum uterine stimulation. This value was obtained from standard dose-response curves established for estradiol-17β. The per cent suppression of testicular growth was calculated on the basis of the difference in degree of growth of testes from treated *vs* untreated controls. Esterification of the parent ethinyl-steroid(1) was effected by reaction with the appropriate acid chloride or anhydride in pyridine solution. The crystalline compounds gave analysis in good agreement with theory and possessed optical rotations within the expected range.

Results. Dose-response relationships for estradiol-17β in UGSt and TGSp assays are shown in Table I. The degree of response in the two tests was proportional to dose. Con-

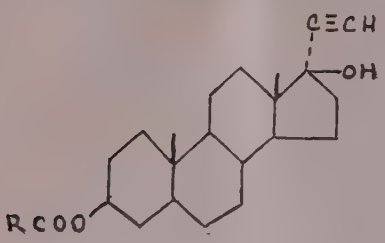
TABLE II. Comparison of Uterine Growth Stimulating (UGSt) and Testicular Growth Suppressing (TGSp) Activities of Various Diol Steroids.

Compound	A UGSt activity ED_{50},* mg/kg/day	B TGSp activity ED_{50},† mg/kg/day	Quotient B/A
Δ^5-Androstene-3β,17β-diol	8.2	47.0	5.7
17α-Ethinyl-Δ^5-androstene-3β,17β-diol	4.75	5.8	1.2
17α-Ethinylandrostane-3β,17β-diol	5.25	6.5	1.2
Estradiol-17β	.0008	.055	68.
17α-Ethinylestradiol-17β	.00045	.0037	9.

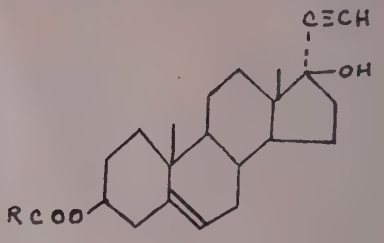
* Dose required to effect 50% of maximum growth of uterus of infantile rats following daily subcut. inj. for 3 days. Estradiol-17β was employed as standard reference.

† Dose required to effect a 50% suppression in normal growth of the testes of immature rats following daily subcut. inj. for 5 days.

TABLE III. Comparison of Uterine Growth Stimulating (UGSt) and Testicular Growth Suppressing (TGSp) Activities of Ethinyl-diol Esters.



Series I



Series II

R	Series	M.P., °C, cor.	A UGSt activity ED_{50}, mg/kg/day	B TGSp activity ED_{50}, mg/kg/day	Quotient B/A
I $-CH_3$	I*	201.2–204.1	.96	3.8	3.9
II $-CH_2CH_2CH_3$	II	115.4–117.0	2.2	4.75	2.2
III $-(CH_2)_5CH_3$	I	89.8– 91.4	8.0	15.0	1.9
IV $-CH_2CH_2COOH$	II	203.0–204.4	16.0	11.8	.74
V $-CH_2$ (S)	II	191.8–194.8	13.0	9.0	.69
VI $-CH_2CH_2$ (S)	I	91.8– 94.2	31.0	17.5	.56
VII $-C(CH_3)_2-CH_3$	I	216.2–218.0	2.7	4.0	1.5
VIII $-(CH_2)_2CH(CH_3)_2$	II	135.6–138.4	5.2	6.1	1.2
IX $-CH(CH_2CH_3)_2$	II	171.4–173.8	21.2	10.5	.49
X $-CH_3$	II*†	171.3–172.9	1.25	6.25	5.1
XI $-CH_2CH_3$	II†	149.7–152.2	3.75	3.75	1.0
XII $-CH_2CH_2$ (S)	II†	115.2–116.6	9.0	7.25	.8

* *Cf* Ruzicka *et al.*(1). † Aqueous suspensions.

siderably more estradiol was required to suppress testicular growth than to stimulate uterine growth. The TGSp ED_{50} was found to be 68 times larger than the UGSt ED_{50}. Although Δ^5-androstenediol was greatly less active mg per mg than estradiol (Table II), the UGSt/TGSp dose ratio was only about 1/12th that determined for the natural estrogen. 17-Ethinylation of estradiol and Δ^5-androstenediol resulted in increased UGSt and TGSp activities. The increment in TGSp activity effected by this chemical modification was much greater than was the increase in UGSt activity, resulting in a considerably lower UGSt/TGSp dose ratio. The extent to which UGSt and TGSp activities as well as dose ratios are altered by esterification of the ethinyl-diol steroids together with the melting points for each compound is shown in Table III.

Within the series of ethinyl-diol esters, the 3-acetate (I) possessed 5 times more UGSt activity than its corresponding alcohol. In-

creasing the size of the ester group resulted in proportionate decrease of this activity. Thus, the 3-heptanoate (III) exhibited approximately ⅛ and the 3-cyclohexylpropionate (VI) approximately 1/32nd the uterine growth stimulating capacity of the 3-acetate (I). A similar structure-activity relationship was obtained for these esters in regard to testicular growth suppressing activity. The 3-acetate of ethinylandrostanediol (I) and the aqueous suspension of the 3-propionate of ethinyl-Δ^5-androstenediol (XI) were the most active in the series. The TGSp activity diminished as the size of the ester group was increased above 3 carbon atoms, but this loss of inhibition was relatively less than was the decrease in UGSt activity. Several of the high molecular weight esters possessed lower UGSt/TGSp ED_{50} ratios than numerous esters lower in the series. The extent to which this dose ratio was affected by enlarging the ester group is shown in the last column of Table III. The α-ethylbutyrate of ethinylandrostenediol (IX) exhibited the lowest UGSt/TGSp dose ratio. This compound possessed 1/22 the UGSt activity of the 3-acetate (I) but was more than ⅓ as active in suppressing testicular growth.

A comparison of activity ratios for the acetate (X), propionate (XI) and cyclohexylpropionate (XII) of ethinylandrostenediol, when administered as aqueous suspensions (Table III), shows that here also the UGSt activity falls off much more abruptly with increasing size of the ester group than does the TGSp activity. Whereas the 3-propionate (XI) was ⅓ as estrogenic as the 3-acetate (X), it was nearly twice as active as the latter in suppressing testicular growth. It should be noted that the aqueous suspension of ethinylandrostenediol 3-cyclohexylpropionate (XII) was considerably more active in the two tests than was the oil solution of the corresponding ester in the ethinylandrostanediol series (X).

It has been found that aqueous suspensions of 3-cyclohexylpropionate (XII) are slowly absorbed from sites of injection and provide extended duration of activity. This compound is well tolerated by tissues and is being examined clinically under the name "ethandrostate" as a possible inhibitor of pituitary gonadotrophin. When administered subcutaneously to mature male rats daily for a period of 2 weeks this ester produced 50% of castration atrophy of the seminal vesicles at dose levels which were 0.3 and 0.6 the estrogenic ED_{50} (UGSt) unit for the oil solution and aqueous suspension, respectively.

Discussion. These observations, in addition to pointing out structure-activity relationships, serve to indicate that a particular chemical change of a biologically active compound does not necessarily modify all activities manifested by that compound to the same degree. The finding that 17-ethinylation increased the TGSp activity of estradiol and Δ^5-androstenediol considerably more than it did UGSt activity, and that certain large molecular weight esters of ethinylandrostanediol and its Δ^5-analog brought about a greater loss of UGSt than TGSp, illustrates altered UGSt/TGSp dose ratios due to different increments of activity in one case and to different degrees of loss of activity in the other case. Several factors suggest themselves as contributory to such effects. A particular chemical change may modify the manifestations of an active agent by altering its rate of absorption from the site of injection, by changing its rate of degradation by tissues, by modifying its rate of entry into the responsive organ or tissue or by any combination of the three. Which of these factors is operative in regard to the compounds studied and reported here has not been established.

The observation that considerably more estradiol-17β was required to suppress testicular growth than to stimulate estrogen sensitive tissues of female immature rats is in accord with earlier reports(8,9). Byrnes and Meyer (10), however, have reported that significant suppression of ovarian growth is effected by subestrogenic doses of estradiol administered daily to 70 g intact female rats for 10 days. Greep and Jones(9), on the other hand, did not observe ovarian regression with less than fully estrogenic doses of estradiol benzoate administered daily for 45 days to rats of similar age and weight. Although the literature

is not definitive in respect to the relative sensitivity of the male and female pituitary to estrogens, there is suggestion that the pituitary of the adolescent male rat is somewhat less sensitive to estradiol than is the female rat pituitary. It should be pointed out that 35-40 g male rats were used in the studies reported here because of the satisfactory dose response of the testes to estrogen, in contrast to the inadequate ovarian changes induced by estrogens in intact rats of this age. Pincus and Werthessen(11) noted that the ovarian response in comparable rats was a function of duration of dosage rather than size of dose. hence we deemed immature female rats unsuitable for assay of suppressive activity.

The much lower UGSt/TGSp dose ratios obtained for several high molecular weight esters of ethinylandrostanediol and its Δ^5-analog than obtained for estradiol or ethinylestradiol suggests that these esters may be relatively more specific than the natural estrogens as inhibitors of pituitary gonadotrophic function. This possibility is being explored in a variety of appropriate follow-up experiments.

Summary and conclusions. (1) The biological activity of ethinylandrostanediol and its Δ^5-analog has been found to be predominantly estrogenic rather than androgenic. The relative testicular growth suppressing (TGSp) activity effected by these ethinyl-diols was considerably greater than observed for equivalent estrogenic doses of estradiol-17β or ethinylestradiol. (2) Low molecular weight esters (less than 6 carbon atoms) of these non-phenolic steroids were more active than their parent alcohols, but the degree of estrogenicity (UGSt) diminished more abruptly than did TGSp activity as the size of the ester group was increased. Of the compounds tested, the 3α-ethylbutyrate of ethinyl-Δ^5-androstenediol effected the greatest degree of testicular growth suppression (TGSp) per unit of estrogenicity (UGSt-ED_{50}), when 35-40 gram immature intacts rats were used.

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Relation of Gonad Hormones to X-Irradiation Sensitivity in Mice.* (22494)

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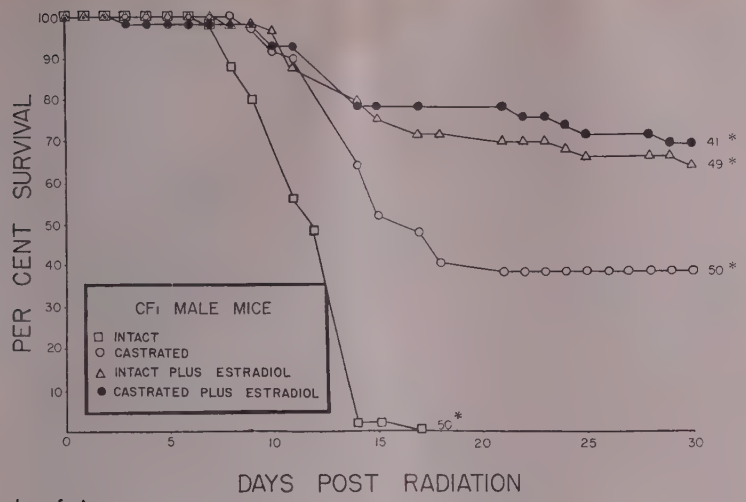
It has been reported(1-5) that a single injection of estradiol benzoate 10 days before an LD 50/30 day exposure to whole body x-rays will increase the resistance of the mouse to the exposure. The present study was designed to determine what effect estradiol benzoate would have in the intact as compared with the castrated male mouse when each is exposed to the LD 50/30 day level of whole body x-rays.

Materials and methods. Ninety-one mature CF_1 male mice were castrated by surgical excision. Aureomycin was sponged over the

* Based on work performed under contract for the Atomic Energy Commission.



* Number of mice.

FIG. 1.

wound to prevent subsequent infection. The mice were then allowed one week to recover fully from the operation at which time 41 castrates and 49 normal, intact males of the same age were each injected intraperitoneally with 0.166 mg of estradiol benzoate in 0.1 cc of sesame oil. Fifty other castrates and 50 intact mice, all of the same age, received no injections. Ten days after hormone injection all of the mice were exposed to 625 r whole body x-irradiation delivered by a Quadrocondex constant potential x-ray machine run at 210 KVP and 15 ma. The rays were selectively hardened by filtration with 0.28 mm Cu and 0.50 mm Al. The distance to the center of the body of each mouse was 30 cm and the output of the machine 271 r/min in air at that position. The mice were irradiated in a plastic cage measuring 14.5 cm in diameter and 4 cm in depth. Lethality data were collected for a period of 30 days.

Experimental. When the percentage survival is plotted against days post-irradiation it is found that the intact male controls, which received no hormone injection, all died by the 17th day. This is not surprising because the dose chosen, 625 r, is LD 50/30 for this species but includes the females which are more radioresistant than the males(6). This dose must then be considered as minimal LD/100/30 days for the adult male CF_1 mouse. At 30 days post-irradiation some 38% of the castrated males were still alive indicating an adverse effect of the presence of the male hormone, testosterone. Maximum survival was found in the castrated males which had also been injected with estradiol benzoate. Of these, 68.3% were still alive. The normal, intact males which had been injected with this hormone showed a survival which was almost as good, namely 63.3%. Thus, the injected female hormone must have counteracted almost entirely the adverse effects of the male hormone.

Discussion. It has been reported that in order to have any protective effect this female hormone, estradiol benzoate, must be injected a number of days before the irradiation(1-5). According to Patt(2) estradiol exerts the maximum protective effect when it is given 10 days before x-irradiation. Patt has also stated that testosterone is ineffectual in protecting mice against irradiation. The above data, relative to the intact mouse, indicate that testosterone is probably deleterious in so far as resistance to x-irradiation is concerned, since all such animals died by the 17th day whereas some 38% of the castrated males, without the presence of the male hormone, survived the 30 day period. In this study

the estradiol exerted its maximal protection when coupled with castration of the male and the consequent removal of the male hormone. Estradiol in the body of the male appears to overcome the deleterious effect of testosterone to a large extent. The intact males which received estradiol showed survival of only 5% less than the castrates plus estradiol. This implies the greater potency of the female as compared with the male hormone, at least in the concentrations available.

Graham and Graham(3) suggested that the resistance to radiation is not related to a simple sex hormone activity. They feel that some extra-gonadal system may be stimulated and protection results. Patt *et al.*(2) suggest that the protective action of estrogens is mediated through the adrenals whereas Ellinger(5) expands this hypothesis stating that "the effect of estrogens may well be mediated by the spleen which is recognized to be under the influence of the adrenal cortex and the functional status of which appears as of great importance for the radiosensitivity in total body irradiation." Bethard, Simmons, and Jacobson(4) report that the survival of mice given estrogens, followed by irradiation with spleen shielding, was greater than when either method was used alone.

Summary and Conclusions. The differential in survival of male and female mice, when exposed to whole body x-irradiation, seems to be due in part to the gonadal hormone present. Intact males, with the normal quota of testosterone, have less survival value than do castrate males. Further, castrate males injected with the female hormone, estradiol benzoate, exhibit maximum survival, approaching that of the intact female.

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Development of Heterotypic Combinations of Dissociated Embryonic Chick Cells.* (22495)

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Some time ago, attempts were initiated to study the behavior *in vitro* of dissociated embryonic chick cells(1-3). Suspensions of viable cells were obtained by disintegrating organ rudiments of the early chick embryo into their constituent cells, using a procedure based on treatment with trypsin. It was found that, under suitable conditions of cultivation, the discrete cells re-aggregated into clusters, re-established a tissue-like association, and subsequently resumed their characteristic histotypical development. Thus, aggregates of limb-bud mesoblastic cells gave rise to cartilage and muscle, and aggregates of mesonephric cells to characteristic nephric tubules and vesicles. These observations were in line with the results of investigations by Weiss and coworkers(4-6) on the fate of embryonic cells in suspension introduced into embryos or grown *in vitro*. The combined evidence strongly indicated that, under the conditions explored, the dissociated cells retained their ability to restore the special histogenetic pattern of their erstwhile associa-

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tion. In most of these experiments, both the identity and proportions of cells in the suspension were the same as in the original tissue. They provided, therefore, little information as to how qualitative and quantitative variations in the original composition of the cell population might affect the outcome. The pertinence of these questions was stressed by diverse observations on chick cell aggregates(2, 5-7), suggesting that cells of different kinds, intermingled in the same suspension culture, tended to become grouped according to kind. Phenomena like these could evidently not be studied in suspensions containing essentially one major cell type only ("isotypic" suspension); their elucidation required a system consisting of 2 or more different cell types ("heterotypic" suspension) clearly distinguishable by their appearance and histogenic properties. The cellular combinations arising in such heterotypic mixtures proved suitable for the study of cellular properties and activities, operative not only in the formation and the development of aggregates, but also in more general histogenetic processes. Of the numerous factors that might affect the final outcome of cell aggregation, the following groups of variables should be considered: 1. The origins and types of cells; their age; proportions of the component types in a given mixture. 2. The physical, chemical and immunological properties of the substrate and the nutrient(8). 3. Cellular interactions; rates of mitotic activity; metabolic activities of the cells; production of extracellular materials or structures. These factors, and others, may affect each or all 3 major steps in the development of aggregates, which are: a) the formation of the primary cluster, *i.e.*, the aggregation of cells by random encounter, directional migration, surface interaction, contraction of extracellular matter; b) organization within the aggregate, either by preferential proliferation and development of some foci with the suppression of others; or by regrouping of the cells within the initial cluster; in either case, the resulting pattern might or might not be identical with the original one; c) the development of the aggregate as an "organoid" system with the possible occurrence of field phenomena, metabolic or diffusion gradients, tissue interactions, etc.

The present report summarizes some observations on a relatively simple heterotypic system, consisting of suspensions of combined mesonephric and limb-bud cells of the chick embryo. Future reports will deal with more complex homologous and heterologous combinations.

Material and methods. Chick embryos from stage 16 (Hamburger) to 5 days' incubation were used. The isolation of tissue blastemas and preparation of cell suspensions and cultures were done as previously described(1), with the following modifications. Final dissociation of the tissue, following treatment with trypsin, was done after rinsing and transfer of the still compact fragments into the culture medium. Stickiness, although rarely a problem with this material, was avoided by keeping the rinsing solutions at pH 7.2-7.6(9). Different concentrations and proportions of cells in the iso- or heterotypic suspensions were obtained by dissociating varying numbers of rudiments, or fractions of a rudiment, in identical quantities of medium. Aliquots of suspension in a mixture of embryo extract (10-12-day embryos), chicken serum and Earle's saline in equal amounts, were placed in hollow ground slides, sealed and incubated. Aggregates that formed during the following 48 hours were then transferred for further cultivation by Fell's watch-glass procedure. The results to be reported are based on a total of 280 cultures, studied in the living and preserved state. The following terms will be used: (1) *isotypic* and (2) *heterotypic,* to designate suspensions consisting of (1) predominantly one type of cell and (2) two or more cell types; (3) *isochronic* for cells obtained from embryos of equal age; (4) *heterochronic* for cells from embryos of different ages.

Aggregates of lateral mesoderm cells from embryos of stage 16. The lateral mesoderm from embryos of ca. 54 hours' incubation representing prospective limb-bud and nephrogenous tissues, was dissected and dissociated either in a single piece or following separation into the respective organ-forming regions. At

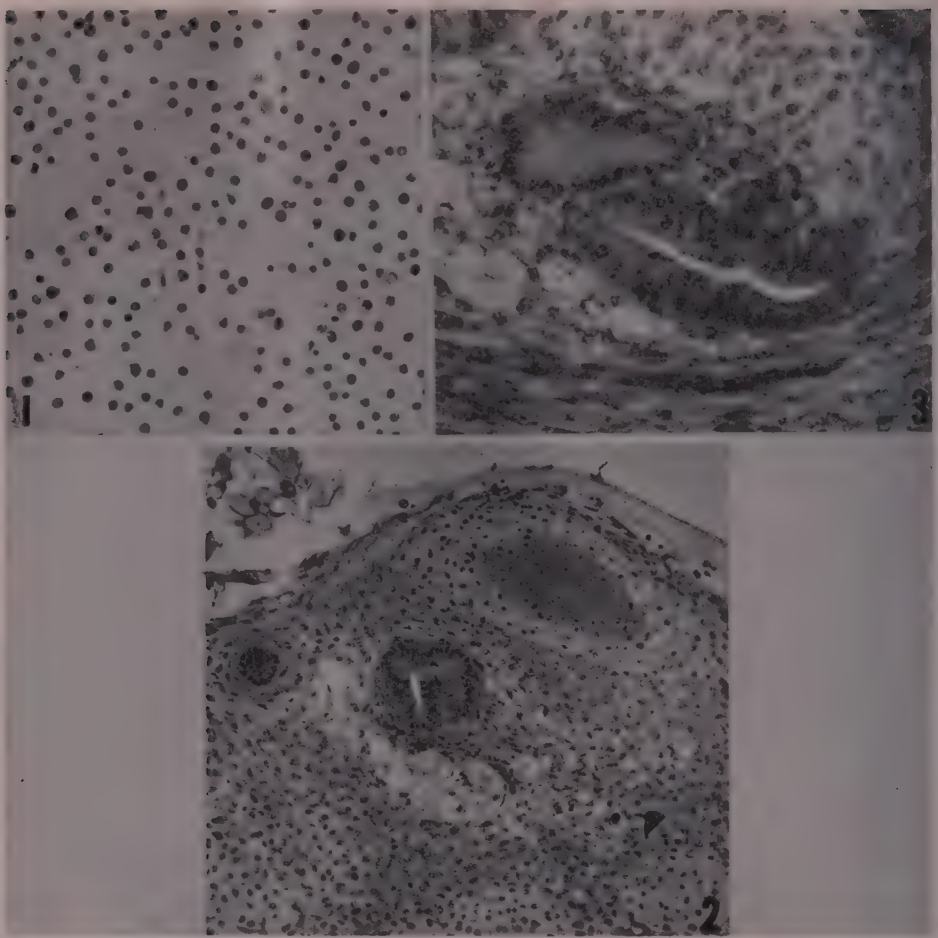

FIG. 1. Cell suspension of lateral mesoderm from embryos of stage 16. ×160. Fixed in formalin-acetic vapour; hematoxylin.

FIG. 2. Section of an aggregate of cells as in Fig. 1, cultured for 6 days on a plasma clot and showing cartilage, nephric tissue, epidermal structures and keratin, fat-cells and macrophages. × 160. Zenker's fix.; hematox.-Biebrich's.

FIG. 3. Same as Fig. 2, showing cartilage, nephric tubules and myoblasts. ×280.

this stage, these regions show no clear structural indication of their subsequent development. The resulting aggregates were grown for 6 to 8 days, and, when examined histologically, were found to have developed in accordance with their normal fate in the embryo: The prospective limb-bud cells had formed cartilage, while nephrogenous cells developed tubules and vesicles. In aggregates formed in the composite suspensions of the whole mesodermal ridges, cartilage and nephric tissue developed side by side (Fig. 1-3). In some cases, myoblasts in the early stages of cytodifferentiation were present. Epidermal cells that had been included in the suspensions gave rise to keratinized epidermal pearls and layers. It was thus established that, even at this early stage of development, dissociated and intermingled cells were able to reconstitute distinct and characteristic tissue fabrics and that their subsequent differentiation followed the type-specific course evidently initiated in them prior to dissociation.

Aggregates of isochronic limb-bud and mes-

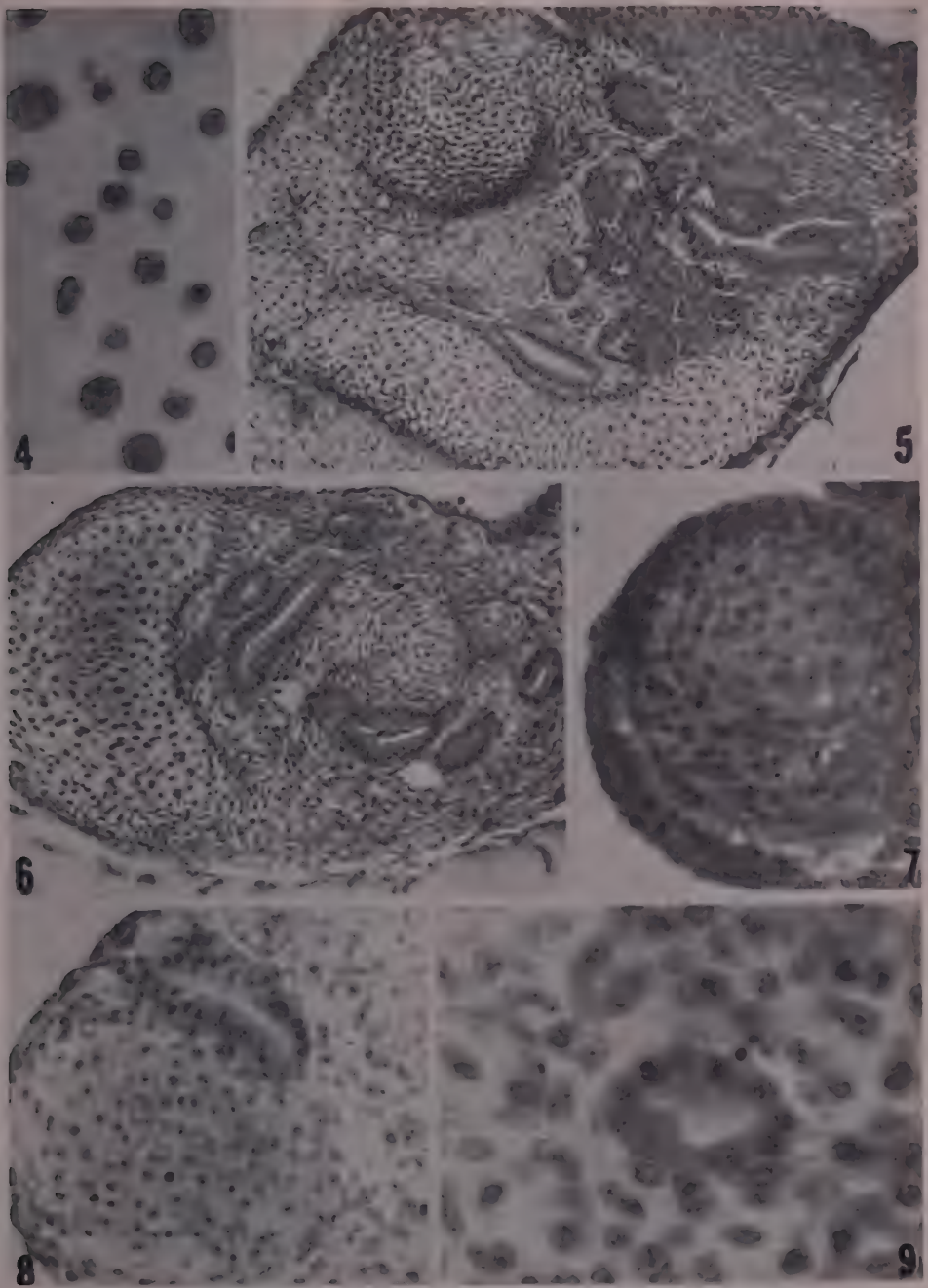

FIG. 4. Suspension of mixed mesonephric and limb-bud cells from a 4-day embryo. ×480.

FIG. 5. Section of an aggregate of cells as in Fig. 4, following 7 days in culture; cartilage, nephric tissue, myoblasts and fibrocytic stroma. ×120.

FIG. 6. Section of an aggregate of 3½-day mesonephric and 5-day chondrogenic cells in equal proportions, cultured for 7 days. ×160.

FIG. 7-9. Parts of section through aggregates of mesonephric and chondrogenic cells to show close association between the two resulting tissues. ×260, 220, 460.

onephric cells from older embryos. This series consisted of aggregates formed in suspensions of combined mesonephric and chondrogenic cells (in some cases whole limb-buds were used) of identical ages, from 3, 4, and 5-day embryos. Both types of tissues then reappeared in these heterotypic aggregates, following 4 to 7 days' cultivation, in their characteristic differentiated form (Fig. 4,5). The progress of differentiation varied according to the types and ages of the precursors. Chondrogenesis at a given total age (age at explantation plus length of cultivation) compared favorably with that in embryos of comparable age. By contrast, the differentiation of nephric tissue lagged behind its progress in the embryo. In aggregates formed in suspensions including myogenic cells, the identified myoblasts were never as numerous as could have been expected from their original proportion. They rarely developed beyond the trapezoid or early spindle-shaped stage (2). This confirmed prior observations *in vitro* that, in the presence of chondrogenic cells, myoblasts did not readily attain advanced differentiation, unless present in proportions exceeding those in the normal limb-bud(3). Yet it was shown(2) that the cause of this impairment did not reside within the explanted myoblasts as such and that given the right conditions they would readily differentiate into muscle cells(10-13). In contrast with other tissues, epidermal cell-groupings, whenever present in the aggregates, showed a precocious formation of keratin as compared with the skin of embryos of equivalent total age. This acceleration has been noted by others(14,5).

Aggregates of heterotypic cells in varying ratios. Considerable evidence exists to suggest that there is a limiting mass minimum for tissue explants, below which continued morphogenesis and development will not occur(15,16). The following observations indicate that in heterotypic systems, histogenesis may be affected, above the level of the limiting mass minimum, by the ratios of cells of different types present in the mixtures. Results obtained by varying the proportions of mesonephric and chondrogenic cells from 4-day embryos illustrate this point. To aliquots of a suspension of chondrogenic cells, mesonephric cells were added in concentrations decreasing serially by a factor of 2. The controls were run as a parallel series of cultures. Examination of the resulting aggregates, following 6 days' cultivation, showed clearly that the lower the proportion of mesonephric cells in the initial suspension, the lower was the frequency of appearance of nephric structures and the smaller was their average size. Ultimately, a certain liminal proportion was reached, below which no recognizable nephric structures were found, although scattered epithelioid cells and patches were in evidence. In the absence of epidermal or other epithelial admixtures, these cells were evidently nephroblasts that had not reconstituted a tissue fabric. By contrast, an equal amount of mesonephric cells cultured separately in the same quantity of medium reaggregated into characteristic tubules. It was not, therefore, the low absolute number of mesonephric cells that interfered with the expression of their typical histogenetic activity, but rather their association with an apparently excessive amount of cells with a basically different histogenetic pattern. Comparable experiments were made with a graded series of decreasing proportions of chondrogenic cells, combined with constant amounts of mesonephric cells. Here again, the size and the number of cartilage foci decreased in the lower concentrations of chondrogenic cells, and below a certain liminal concentration no cartilage appeared in the cultures during 6 days' cultivation. Such cultures showed normal nephric tissue and fibroblastic cells. And yet, a similar quantity of chondrogenic cells in an isotypic suspension reaggregated and did develop into cartilage. In fact, it was noted by Fell(17), as well as in this study, that even quite minute, isolated fragments of chondrogenic tissue, consisting of 30 to 40 cells, if grown as such under suitable conditions, differentiated readily into cartilage.

This evidence seems to suggest that the manifestation of histogenetic activity in a heterotypic suspension might be profoundly affected by the relative proportions of the cells in the original mixture. The mere presence of cells of a certain type in a heterotypic sys-

tem does not, therefore, insure their expression as a typical tissue. Unless present above a liminal proportionate concentration, they do not give rise to a coherent tissue fabric. It may be assumed that these liminal values vary for different cell types as well as for each type at different stages of development and in different heterotypic combinations.

Heterotypic-heterochronic aggregates. Such aggregates were obtained by intermingling mesonephric and chondrogenic (or limb-bud) cells from 3- and 5-day embryos. Whenever the cell ratios were above the respective liminal values for histogenetic activity, cartilage, nephric tissue and epidermis (if such was originally included) appeared regularly in the cultures, following 4 to 6 days' cultivation (Fig. 7). Histogenesis and the ultimate appearance of the tissues were normal. Taking into account the expected differences in rates of development of tissues in the heterochronic combinations, no essential difference was noted, within the tested range, between the development of the isochronic and the heterochronic aggregates. The different types of cells, originally incorporated in the suspensions, appeared in contiguous or scattered groupings, each manifesting the typical structural properties of the tissue of origin.

It should be mentioned at this point that Trinkaus and Groves(7) reported an inhibition of cartilage formation in aggregates of mixed 3-day mesonephric and 5-day limb-bud cells, as well as in aggregates of limb-bud cells only. In view of the theoretical considerations raised by the authors, it should be stressed that in our experiments with the same type of material we have found no evidence for this effect.

Regional groupings of cells in heterotypic aggregates. As already pointed out, the reaggregated cells, in the fully developed heterotypic aggregates, appeared in histotypically distinct, major groupings. These easily identifiable clusters of cartilage, nephric or epidermal structures were interconnected by a stroma of fibrocytes, mainly of connective tissue and myoblastic origin, sometimes including fat-cells, macrophages and possibly also cells of chondrogenic and mesonephric origin. In their chaotic architecture, the aggregates strikingly resembled teratomatous structures (6). On closer scrutiny, small groups of cells were found scattered in various locations in close association with cells of other types. Small groups of nephric or epidermal cells were frequently observed either tucked in between masses of cartilage (Fig. 8) or right inside the chondrified tissue (Fig. 9). On some occasions, a layer of nephric cells was found to have enveloped a chondrified core (Fig. 7). In spite of their close association, it was possible to identify the different components with a fair measure of certainty. It seemed, therefore, that the cells had not only preserved their original identity throughout the phase of dissociation and aggregation, but had also re-established specific and selective contiguity, as evidenced by their assortment into type-specific groupings(18,19). Considering the complete initial intermingling of the cells in suspension, their ultimate arrangement suggests the following possibilities to account for the final structure of the aggregate: (a) that it is determined during the initial phase of aggregation by differences in the rates of movement of different cell types, by contact interactions, etc.; (b) that it is due to reorganization within the primary clusters through active cell movements and selective cell groupings; (c) that it is attributable to differential survival and proliferation of the various components; (d) that it is affected by all of these factors. The evidence presented here does not allow a critical evaluation of these possibilities.

Summary. 1. The behavior *in vitro* of dissociated and reaggregated mesonephric and limb-bud cells of the early chick embryo was studied in heterotypic, isochronic and heterochronic combinations. 2. Under the conditions examined, all cell types incorporated in the cell suspensions reappeared eventually in the aggregates to form tissue-like groupings that developed in accordance with their tissues of origin. 3. Manifestation of the characteristic histogenetic activity by cells of a given type in a heterotypic combination decreased in proportion to their share in the total population. If present below a certain liminal proportion, the cells did not reconstitute their typical tissue fabric, although equal

amounts, when cultured separately, would differentiate histotypically.

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Effect of Varying Dosages of Potassium Iodide in Experimental Atherosclerosis. (22496)

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Several investigators have reported that potassium iodide(1,2), organic iodides(3) or desiccated thyroid(3) inhibited formation of atheromatous plaques in cholesterol-fed rabbits. While KI and organic iodides had a tendency to cause hypercholesterolemia, thyroid powder lowered serum cholesterol somewhat. Similar effects of these compounds on serum cholesterol have also been observed in chickens(4), although no anti-atherogenic influence was found. Recently, administration of KI to rats for very short periods was shown to cause a 2-fold increase in serum cholesterol (5).

The present study was designed to examine effects of various dosages of KI as well as of 2 thyroid-active substances (diiodotyrosine and thyroxine) on development of atheromata and on serum cholesterol and b-lipoproteins of rabbits fed cholesterol. The anti-atherogenic effects of large dosages of KI are confirmed.

Methods. Male rabbits of the Dutch belted strain were maintained on a diet of rabbit chow (Wayne Rabbit Ration, Allied Mills, Chicago, Ill.) which had been thoroughly mixed with a suspension of cholesterol in corn-oil so that every 100 g of food contained 2% cholesterol and 6% corn-oil. The basic diet was augmented with amounts of KI or of diiodotyrosine as noted in Tables I and II by spraying the appropriate solution on the pellets while they were tumbled in a concrete mixer. This was done before adding the cholesterol-oil suspension. The thyroxine was administered by the intraperitoneal injection of 0.05 mg, 3 times weekly. After 8 weeks the animals were sacrificed and aortas examined visually for atheromata and graded on a 0-4 scale. Averages for each group are given in Table I. In Exp. 1 and 2 the entire aorta was graded, in Exp. 3 the arch and thoracic portions were graded separately. Sera were assayed for cholesterol(6) and lipoproteins(7) and livers were excised, weighed wet and analyzed for cholesterol content. Average values of these are stated in Tables I and II.

TABLE I. Autopsy Findings on Rabbits Fed Cholesterol Diets Augmented by KI, Diiodotyrosine and Thyroxine.

Group*	No. of animals	Wt change (g)	Atheromata		Serum chol. (mg %)	Liver wt (g)	Liver chol. (%)
Exp. 1							
1% KI	9	—	.55‡		2170	70	2.9‡
B†	10	—	1.55		2260	98	8.3
Exp. 2							
1% KI	8	106	1.00§		3830	—	—
B	14	212	2.70		3150	—	—
Exp. 3			Arch	Thoracic			
.001% KI	10	132	2.20	1.40	2520	89	4.1
.01% "	9	60	2.11	1.44	2610	81	3.9
.1% "	8	−3	1.94	1.00	2490	75	3.6
1 % "	7	−59	.93§	.14	2400	63	2.3‡
DIT‖	10	117	2.65	1.40	2360	67	3.7
T¶	10	−51	1.75	.75	2270	88	4.0
B	7	81	2.43	.93	2800	80	4.0

* All diets contain 2% cholesterol, 6% oil. † B = Basic diet. ‡ $p < .0001$.
§ $p < .001$. ‖ 0.1% diiodotyrosine. ¶ Thyroxine, 0.15 mg/wk, i.p.

TABLE II. Serum Lipoprotein Levels of Rabbits Fed Cholesterol Augmented by KI, Diiodotyrosine and Thyroxine.

Group*	Serum lipoproteins, S_f mg %					Total
	0–12	12–20	20–35	35–100	100–400	
Exp. 1						
1% KI	480	727	528	650	461	2846
B†	165	550	851	650	411	2627
Exp. 2						
1% KI	998	649	616	1064	1209	4536
B	362	750	1223	1309	682	4326
Exp. 3						
.001% KI	261	555	1006	1388	696	4006
.01% "	158	490	864	1000	546	3058
.1% "	208	491	786	1106	816	3407
1 % "	367	464	522	956	1050	3359
DIT‡	186	448	773	1202	829	3438
T§	115	274	675	933	861	2858
B	241	481	845	1301	1073	3941

* All diets contain 2% cholesterol, 6% oil. † B = Basic diet. ‡ 0.1% diiodotyrosine.
§ Thyroxine, 0.15 mg/wk, i.p.

Results. The data show that only large amounts of KI had any significant effect on formation of atheromata. At levels administered, neither diiodotyrosine nor thyroxine inhibited atherogenesis. None of the compounds produced significant hypocholesterolemia, nor was there a distinct lowering in lipoprotein fractions. In this connection it is of interest that the concentration of the cholesterol-rich S_f 0-12 class lipoproteins(8) was higher in the group receiving 1% KI than in controls. Of groups in Exp. 3, that on thyroxine showed lowest cholesterol and lipoprotein levels although atheromata were considerably higher than those in animals being fed 1% KI.

Moses and Longabaugh(9) observed no significant reduction in atheromata when they administered small dosages of KI to growing rabbits. Groups receiving 325 mg KI daily (plus 15 g cholesterol/week) showed somewhat lower average atheromata than did the control group, while the atheromata of the group fed 20 mg KI were more severe. Rosenthal(10), using very small doses of KI (3-7 mg) in cholesterol-fed rabbits found elevated serum cholesterol levels, and more lipid in

aortas of these animals than in the controls. Greatest protection against atherosclerosis was observed when the cholesterol KI ratio was approximately 2:1. Turner(1,2) fed equal amounts of cholesterol and KI, and Page and Bernhard(3) fed approximately equal amounts of cholesterol and diiodoricinsterolic acid. Moses and Longabaugh(9) employed ratios of 7:1 and 100:1, with the former giving relatively greater protection.

In Exp. 3 there was a slight weight loss in groups receiving 0.1% KI, 1% KI and thyroxine, but the overall weight difference in all groups was small. Firstbrook(11) has pointed out the high correlation between relative weight gain and severity of lesions in experimental atherosclerosis in rabbits, but the gains and losses recorded in Exp. 3 are too small to have affected the results. Both KI and control groups gained in weight, and there seemed to be no correlation in individual animals between atheromata and weight change. The 1% KI diet, while causing no hypocholesterolemia, lowered average liver cholesterol content significantly.

Summary. 1. Groups of rabbits were fed a 2% cholesterol diet augmented with 0.001%, 0.01%, 0.1% and 1% potassium iodide, 0.1% diiodotyrosine, and one group received 0.15 mg of thyroxine weekly by intraperitoneal injection. 2. Serum cholesterol and lipoprotein levels of all animals were elevated, but atheromata were significantly reduced in those on diet containing 1% KI. The S_f 0-12 lipoprotein levels were also higher in animals of this group than in controls, but on the whole levels of liver cholesterol were lowest.

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Cytopathogenic Agent Resembling Human Salivary Gland Virus Recovered from Tissue Cultures of Human Adenoids. (22497)

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Smith(1) recovered a cytopathogenic virus from a human salivary gland demonstrating intranuclear inclusions characteristic of the human salivary gland virus. Subsequently, Weller(2) isolated a similar virus from a liver biopsy from an infant with a clinical diagnosis of cytomegalic inclusion disease. During the course of studies in this laboratory of the presence of adenoidal-pharyngeal-conjunctival (APC) viruses(3-5) in human adenoids, 3 strains of an intranuclear inclusion body-producing virus were isolated from adenoid tissue cultures in which the fibroblasts underwent spontaneous degeneration. This report describes some of the properties of the virus, the development of a complement fixation test, and the serological relationship of this virus to the strains isolated by Smith and Weller.

Materials and methods. Adenoids were obtained from children undergoing tonsillectomy-adenoidectomy at the Children's Hospital, Washington, D.C., and the Clini-

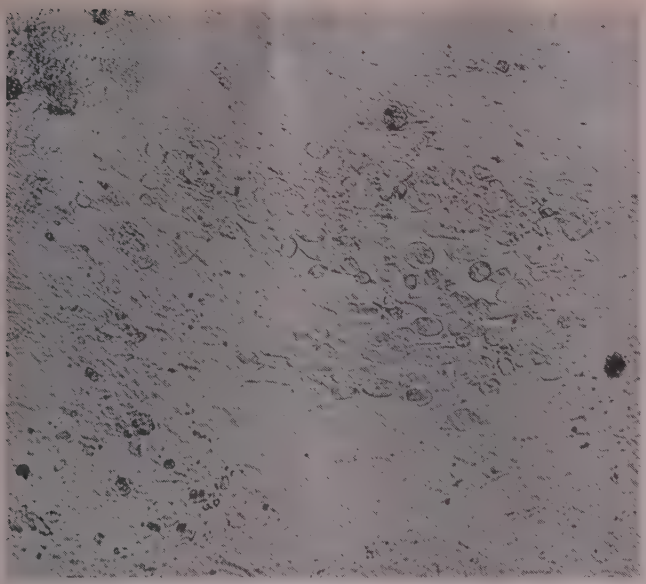

FIG. 1. Ad. 182 strain. First passage, adenoid tissue, 14th day after inoculation. Focal area of rounding in sheet of fibroblasts. Unstained, ×125.

cal Center of the National Institutes of Health. Roller tube cultures of adenoids and embryonic tissues were prepared as described previously(3). Trypsin-dispersed cultures of human fibroblasts were prepared from embryonic skin-muscle, using a modification of the Youngner procedure(6). These cells were maintained in serial passage by Microbiological Associates Inc., Bethesda, Md., from whom the majority of cultures were obtained. The cells were grown directly on glass in stationary tubes and 32 oz. bottles, using a growth medium consisting of 10% to 20% human serum in Eagle's basal medium (7). Prior to inoculation, the cultures were washed four times with Eagle's medium and changed to a maintenance medium consisting of 5% horse serum in Eagle's medium; tube and bottle cultures received 1.0 and 40.0 ml respectively. All media contained penicillin (50-250 u/ml) and streptomycin (50-250 μg/ml). Nutrient fluids of tube cultures were changed once or twice weekly; bottle culture fluids were changed less often. Complement fixation (CF) tests were performed with a modified Bengston procedure(5). Neutralization tests were done in trypsinized human embryonic fibroblast cultures, using the neutralization procedure employed in APC virus studies(5). Seed viruses consisted of freshly prepared tissue suspensions from cultures showing severe cytopathogenic changes; lightly centrifuged preparations gave more reproducible results than uncentrifuged preparations, but the incubation period of the cytopathogenic effects was often unduly prolonged. A serum was considered positive if in two consecutive readings made several days apart, the cytopathogenic changes were less than half the degree of those in the virus control tubes.

Isolation of Strains. The 3 strains were recovered from adenoids taken from an 11-year-old boy (Ad. 162), a 7-year-old girl (Ad. 169) and a 6-year-old girl (Ad. 182). After 22 to 51 days in culture, epithelial sheets in several tube cultures of each adenoid underwent spontaneous degeneration characteristic of APC virus, and APC viruses were demonstrated in the culture fluids. Other culture tubes from each adenoid did not show APC virus effects, and after 71, 64, and 34 days in culture, respectively, one culture of each set showed focal areas of degeneration in the fibroblasts. Subsequently, one or 2 additional tube cultures of each adenoid developed identical

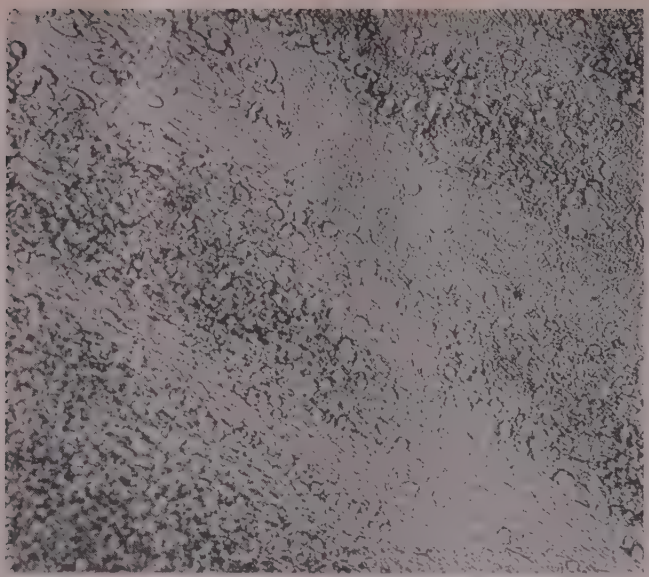

FIG. 2. Ad. 169 strain. Second passage, tonsil tissue, 56th day after inoculation. Numerous oval foci with dense pigmentation. Unstained, ×100.

changes, in one instance after 120 days in culture. The changes consisted of sharply delineated oval areas of clear, smoothly outlined oval or round cells. As the foci enlarged, the central cells became necrotic and densely pigmented, eventually leaving a granular mass in the center of the focus, surrounded by a thin border of rounded cells. Progression of the effects was very slow, usually requiring six to eight weeks for complete destruction of the fibroblasts. First passage was carried out by scraping off the involved tissues, grinding in a tissue grinder, and resuspending in a small volume of supernatant fluid. This material was immediately inoculated into roller tube cultures of adenoids and tonsils showing predominantly fibroblastic outgrowth, no other fibroblast cultures being available. Cytopathogenic effects identical to those in the original cultures developed after an incubation period of 7 to 50 days (Fig. 1 and 2). All three strains were carried through at least 2 serial passages by this method. Table I shows a representative passage series of the Ad. 169 strain. At the fourth passage, the incubation period shortened markedly, and the virus passed well subsequently.

Properties of Ad. 169 Strain. Cytopatho-

TABLE I. Representative Passage Series of Ad. 169 Strain.

Passage No.	Tissue	Type of culture	Day of first CPE	Day when CPE became generalized	CF antigen titer (cell susp.)
	Ad. 169	Roller tube (explant culture)	64		
1	Adenoid	Roller tube	7	45	
2	Tonsil	*Idem*	13	43	
3	Human embryo skin	"	20	61	>1
4	*Idem*	"	2	12	
5	Trypsinized H.E. skin-muscle	Bottle	4	6	6
6	*Idem*	"	4	9	6
7	"	"	3	5	2
8	"	"	5	11	>8
9	"	"	<5	10	16
10	"	"	1	4	

TABLE II. Virus Activity and CF Antigen Titer at Intervals after Inoculation of Bottle Culture with 8th Passage Ad. 169 Strain.

	Material tested	Time of sampling (days after infection of bottle culture) 3	5	7	9	11	13	15	17	20
Incubation period of cytopathogenic effects (duplicate cultures), days	Culture fluid	>47	10	>48	13	>44	8	7	6	7
		>47	>45	>48	>46	>44	9	7	6	
	Cell susp.					4	1	5	1	4
						4	2	5	1	5
CF antigen titer	Culture fluid	0	0	0	0	0	0	1:1	1:1	1:1
	Cell susp.					1:1	1:2	1:4	1:4	1:8
Cytopathogenic effects in bottle culture		±	+				3+			4+

genic changes were produced in all types of human fibroblast cultures tested, including tonsils, adenoids, foreskins, and embryo skin-muscle. In early passages, the changes were identical to those seen in original cultures; in later passages distribution of round cells occasionally became generalized, and only minimal granulation was observed. Hematoxylin-eosin stained preparations revealed that numerous cells in the foci contained large eosinophilic intranuclear inclusion bodies with margination of chromatin and halo formation. In Giemsa stained cultures, the nuclei containing inclusions also demonstrated one or two deeply basophilic bodies in the clear zone or on the nuclear membrane. In cultures containing both epithelium and fibroblasts, the epithelium remained unaffected, except in rare instances when cells immediately adjacent to affected fibroblasts underwent clumping and inclusion body formation. Cytopathogenic changes were not induced in cultures of HeLa cells, KB cells(8), monkey kidney, or rabbit trachea. Adult and suckling mice injected intracerebrally and intraperitoneally and rabbits inoculated intradermally and onto the scarified cornea showed no sign of illness. The virus passed through a Selas .015 filter (maximum pore diameter 2.8 mu) with no loss of activity, but filtration through a Selas .03 filter (maximum pore diameter 1.2 mu) prolonged the incubation period. Infectivity was destroyed by exposure to 20% diethyl ether for two hours. Aureomycin, 50 μg/ml, did not affect virus activity. There was no loss of infectivity in cell suspensions held for 3 hours at 37°C, room temperature, or 4°C. The virus did not withstand storage for a week at 4°C, and much virus was lost after quick freezing and storage at –20°C or –40°C; slow freezing with storage in a dry ice chest appeared more satisfactory for virus preservation. Supernatant culture fluids from early passages did not contain detectable virus, but in later passages the fluids were generally infectious. Table II demonstrates the pattern of virus release in a bottle culture. Five of 6 bottles tested, in 2 experiments, demonstrated peaks of virus activity on the 5th and 9th days, with markedly prolonged incubation periods of fluids removed on the 3rd, 7th, and 11th days. The occasional variation in incubation period of cytopathogenic changes in duplicate tubes was possibly due to the presence of virus in cell clumps, rather than as a uniform suspension. On the 13th day, regardless of whether the bottle had been partially scraped for testing of cell suspensions, the quantity of virus in the fluid increased markedly, and tended to remain elevated for the duration of the experiment. However, in 3 of the 6 bottles, the virus content of the culture fluid was again very low when sampled after all cells had been affected. The cell suspensions consistently produced cytopathogenic changes after a shorter incubation period than the culture fluids, and complement fixing antigen was likewise present in higher titer in the cells.

Serological Tests. Initial experiments to detect the presence of complement fixing antigen were done by testing culture fluids and cell suspensions against the serums of the children from whose adenoids the new agents had been recovered; a number of preparations gave positive reactions with the serums of the donors of Ad. 162 and Ad. 169, but not of Ad. 182. Known positive antigens were then used

TABLE III. Relationship of Neutralizing to Complement Fixing Antibodies in Human Serums by Age Group.

CF antibody (1:8)	Neutralizing antibody (1:4) 1-5 yr + ± —	18-25 yr + ± —	> 35 yr + ± —
Positive (≥ 1:8)	7 0 0	3 0 0	11 0 0
Partial (1:8 (1-2+)	0 0 1	0 0 0	2 1 0
Negative (<1:8)	0 0 7	0 1 5	3 1 1

to test a number of human serums, and a high titer serum which was available in large quantity was selected as a standard serum for titrating antigens. This serum reacted in CF with antigens prepared from each of the three adenoid isolations. A number of positive human serums were tested against a control antigen consisting of a suspension of uninfected human embryo fibroblasts, and no reactions were obtained. Antigens were routinely heated at 56°C for 15 minutes, since this procedure generally removed anticomplementary activity without affecting antigen titer. CF antigen activity was not affected by storage at −20° for 3 months. The occurrence of CF antibodies in relation to neutralizing antibodies in human serums is indicated in Table III. In children and young adults there was very close agreement between the two tests, but in older adults serums negative in CF were often positive in neutralization. Serums positive in CF were in all instances positive in the neutralization test.

Relationship of Ad. 169 Strain to Other Viruses. Because of their similar cultural behavior, the agents recovered by Smith(1) and by Weller (Davis strain)(2) were compared with the Ad. 169 strain in this laboratory and in that of Dr. Weller; only the results obtained in this laboratory will be mentioned here. The general behavior of the 3 viruses appeared very similar, including incubation periods, patterns and rate of progression of cytopathogenic changes, and presence of virus in higher titer in cell suspensions than in culture fluids. Eight human serums having CF antibody to the Ad. 169 strain, and 8 serums having no CF antibody were tested for neutralizing and/or complement fixing antibodies to the Ad. 169, Smith, and Davis agents (Table IV). The majority of serums tested were from young adults. There was excellent correlation between the tests with the 3 viruses. The same association of antibodies was observed in 6 additional serums on which all tests were not completed. Thus the close cultural and immunological similarity between these strains strongly suggests that they are closely related viruses, and that the Ad. 169 and Smith strains may be identical. Paired acute and convalescent serums from three children with herpetic gingivostomatitis showed antibody rises to herpes simplex virus, but no antibody response to the Ad. 169 virus; a herpes simplex rabbit antiserum with a homologous CF titer of 1:64 gave no CF reaction when tested at a 1:8 dilution against a known positive Ad. 169 antigen. The patterns of cytopathogenic changes produced by the Ad. 169 virus suggested a possible relationship to varicella virus(9); however, in the opinion of Dr. Weller(2), the presence of pigment in the foci and the presence of virus in the culture fluids sharply distinguish the Ad. 169 and Davis agents from varicella. In addition, paired serums from three children with clinical varicella showed no CF antibody response to the Ad. 169 virus. Similarly, paired serums from 10 cases of measles showed no CF antibody rise against the Ad. 169 virus. Because of their recovery from adenoid tissue, it was important to exclude a relationship to the APC virus group. There was no cross reaction between the Ad. 169

TABLE IV. Tests of 16 Human Serums for Neutralizing and/or Complement Fixing Antibodies to Ad. 169, Smith, and Davis Strains.

Ad. 169 CF	No. of serums showing following pattern	Smith CF	Smith Neut.	Davis CF
+	5	+	+	+
(8 serums)*	3	±	+	+
—	7	—	—	—
(8 serums)†	1	±	±	±

* Seven tested in neutralization vs Ad. 169; all positive.

† Ideas all negative.

‡ Serums tested at 1:4 in CF and at 1:8 in neutralization.

+, positive test; ±, partial reaction (1-2+ CF reaction, partial delay in neutralization); —, negative test.

TABLE V. Occurrence of CF Antibodies to Ad. 169 Virus by Age and Sex.

Age group	♂ No. positive/No. tested	♂ % positive	♀ No. positive/No. tested	♀ % positive	Total No. positive/No. tested	Total % positive
Birth (cord blood)					12/17	71
6 wk					5/17	29
6-23 mo	3/15	20	0/ 6	0	3/21	14
2- 4 yr	8/17	47	3/19	16	11/36	31
5- 9 "	4/16	25	7/17	41	11/33	33
10-15 "	10/24	42	12/25	48	22/49	45
18-25 "	33/73	45	19/25	76	52/98	53
> 35 "	11/15	73	31/37	84	42/52	81

virus and APC viruses in CF tests; also, the lack of cytopathogenicity for epithelium and the sensitivity to ether treatment have not been observed with any APC virus.

Distribution of Antibodies to Ad. 169 Strain. Complement fixing antibodies were found in a high proportion of human serums (Table V). Cord blood serums and serums from 6-week-old infants were obtained from the same children. Children 6 months to 15 years of age recently admitted to a foundling home provided the majority of children's serums tested. The serums from young adult populations were obtained from student nurses, pregnant women, military recruits, and male penitentiary inmates. The persons over 35 years of age who were tested were employees or patients at the National Institutes of Health, the latter usually having infectious or malignant diseases. Antibodies were frequently present at birth, were less frequent at 6 weeks, and after the age of 6 months, tended to become increasingly prevalent with age. Because of the differences in composition of the sampled populations, the sex differences cannot be regarded as highly significant. Occurrence and development of CF antibodies were observed in serial bleedings of infants. In addition to cord serum and the 6 week bleeding recorded in Table IV, a number of subsequent serums obtained up to the age of 4 were available on 15 of the children.* Ten of the 15 had antibody in the cord serum; 5 were still positive at 6 weeks, but in all instances the titer was lower than in the cord serum. By the age of one or two years, 3 of the 10 had reappearance of antibodies or a 4-fold rise in titer; in all 3 instances, the first serum obtained after the 6 week bleeding was positive. Of the 5 children with no antibody in the cord serum, all were still negative at 6 weeks; however, one of the 5 became positive by the age of 11 months. Three of the positive children were followed for several years with serial titrations; in all instances, the CF titer decreased markedly within 2 to 3 years. In contrast to the decline in antibodies in the children, titrations of serial serums of 3 adult males indicated that CF antibodies remained elevated for at least 4 to 7 years.

Discussion. Several findings suggest a relationship of the newly isolated agents to the pathological entities of human salivary gland inclusions and cytomegalic inclusion disease, which have been suspected to be manifestations produced by a single virus(10). The recovery of the Smith virus from a salivary gland containing typical nuclear inclusions, and the isolation of the Davis virus from cytomegalic inclusion disease strongly support this hypothesis. Also, the inclusions produced in tissue culture by the Ad. 169 strain were reported by Dr. Henry Pinkerton(11) to be pathognomonic of the salivary gland virus. Finally, the type of rounding produced and the strict tropism for fibroblasts are similar to the effects produced by the mouse salivary gland virus in tissue culture(12).

The close correlation observed here between the presence of complement fixing and neutralizing antibodies in human serums is somewhat unique, and resembles the antibody patterns found with herpes simplex(13). This finding suggests that there is but one serological

* The serums were kindly supplied by Dr. Robert H. Parrott.

type of this agent, and is compatible with the concept that the virus persists in the body as is true of herpes simplex and the salivary gland viruses of rodents. However, inclusions are rarely found in salivary glands of adult humans(14). In view of its recovery from adenoid tissue, it appears possible that the virus may persist in lymphoid tissue.

The age distribution of antibodies and the pattern of acquisition of antibody after birth, as reported herein, is somewhat at variance with the usual concept of human salivary gland virus infection, which on the basis of morphologic studies appeared primarily to be an infection of infancy, with many infections acquired *in utero*(10).

The value of the procedure of growing tissues in culture for unmasking indigenous cytopathogenic agents is exemplified here as in similar studies of APC viruses.

Summary. Three strains of an intranuclear inclusion body-producing virus were isolated from spontaneously degenerating tissue cultures of human adenoids. One strain was studied in detail and appeared to be closely related to or identical to viruses isolated in other laboratories from a human salivary gland and a case of cytomegalic inclusion disease. It seems likely that these agents are representatives of the human salivary gland virus. Antibodies were found in a high proportion of human serums.

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Propagation in Tissue Cultures of a Cytopathogenic Virus from Human Salivary Gland Virus (SGV) Disease.* (22498)

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It is now well recognized that there exist in man, monkeys and several rodents closely related viruses, which lie dormant in the salivary glands, but are capable of causing fatal generalized infections with visceral necrosis (1). A general term for these agents is salivary gland virus (SGV). Each virus or strain of the virus is probably species-specific,

* This investigation was supported by research grant (PHS E-158) from the National Microbiological Institute, of the N. I. H., Public Health Service.

since all attempts to infect animals of one species with virus derived from another species have failed. Serial transmission to animals of the same species has been accomplished with certain rodent strains; in young mice a fatal generalized infection is produced by intraperitoneal injection of infectious material(2). Tissue infection with these viruses results in cellular gigantism, with the formation of nuclear inclusions which, by their huge size, structure and staining characteristics, are set apart from those associated with other known viruses. In man and some other species basophilic inclusions also occur in the cytoplasm of many of the cells which have intranuclear inclusions. The incidence of intranuclear inclusions in the salivary glands of infants and young children, regardless of the cause of death, has been reported from different geographical areas as 10 to 30% in routine autopsies(3-5). The cytologic picture of the infected cells is considered pathognomonic(6), and the diagnosis of generalized human SGV infection is being made confidently and with increasing frequency on morphologic evidence alone; in some geographic areas, it is reported in approximately 1% of all infants and young children dying under the age of 5 years(7,8).

In vitro propagation of the species-specific virus is of obvious importance for further study of the disease. Propagation of the mouse SGV in cultures of mouse tissue has been reported(9). This paper is concerned with the propagation of a cytopathogenic virus from 2 cases of human SGV disease.

Material and methods. Tissue cultures. Pieces of human uterine wall were minced with scissors in Hanks' balanced salt solution and ox-serum ultrafiltrate (2:1). Fifteen to 20 implants were embedded in a thin layer of clotted chicken plasma on the walls of culture tubes (20 x 150 mm). The nutrient medium consisted of Hanks' balanced salt solution and ox-serum ultrafiltrate (2:1), chicken or beef embryonic extract (10%) and inactivated horse serum (10%). One-hundred units of penicillin and 100 μg of streptomycin per ml were added to the medium. The cultures were incubated at 34 to 35°C in a frame revolving 9x per hour, and the nutrient fluid was changed at intervals of 2 or 3 days. The cultures were maintained usually for 10 to 14 days before introducing the virus. *Origin of the Cytopathogenic Virus.* The agents were isolated from tissues obtained at autopsy. To obtain infective material both submaxillary salivary glands were removed at autopsy from infants under 3 years of age, using sterile instruments. One gland was fixed for microscopic study and the other placed in a sterile tube and frozen immediately in dry ice. The first infective agent to be isolated was from a submaxillary salivary gland of a 7-month-old infant dying from adrenal cortical carcinoma. In this case the other gland which was examined microscopically showed many characteristic inclusions, but no visceral involvement was found. The second agent was isolated from the kidney of a 1-month-old infant dying of generalized SGV disease (cytomegalic inclusion disease). The renal tissue was preserved in dry ice for 1 week before it was used for inoculation of cultures. Many characteristic inclusions were present in microsections of the kidneys and other viscera as well as in the salivary glands of this infant. *Preparation of Cultures for Histological Study.* All cultures for histological study were fixed in Bouin's solution and stained with hematoxylin and eosin. Many cultures were fixed and stained in the tubes. These preparations were cleared and preserved permanently in the tubes in xylol. They were studied with the low power objective (10 x). Other cultures were embedded, after fixation, in thin collodion in the tubes. The collodion cast was removed from the tube, cut into pieces of desired size, stained and mounted, according to the technic described by Enders(10).

Experimental. The salivary gland from which the first agent was isolated was ground in a mortar with a small amount of sterile alundum and diluted 1:5 in Hanks' solution and ox-serum ultrafiltrate. Penicillin and streptomycin were added. After centrifugation, 1 ml of the supernatant fluid and 1 ml of nutrient medium, containing twice the usual amounts of embryonic extract and horse

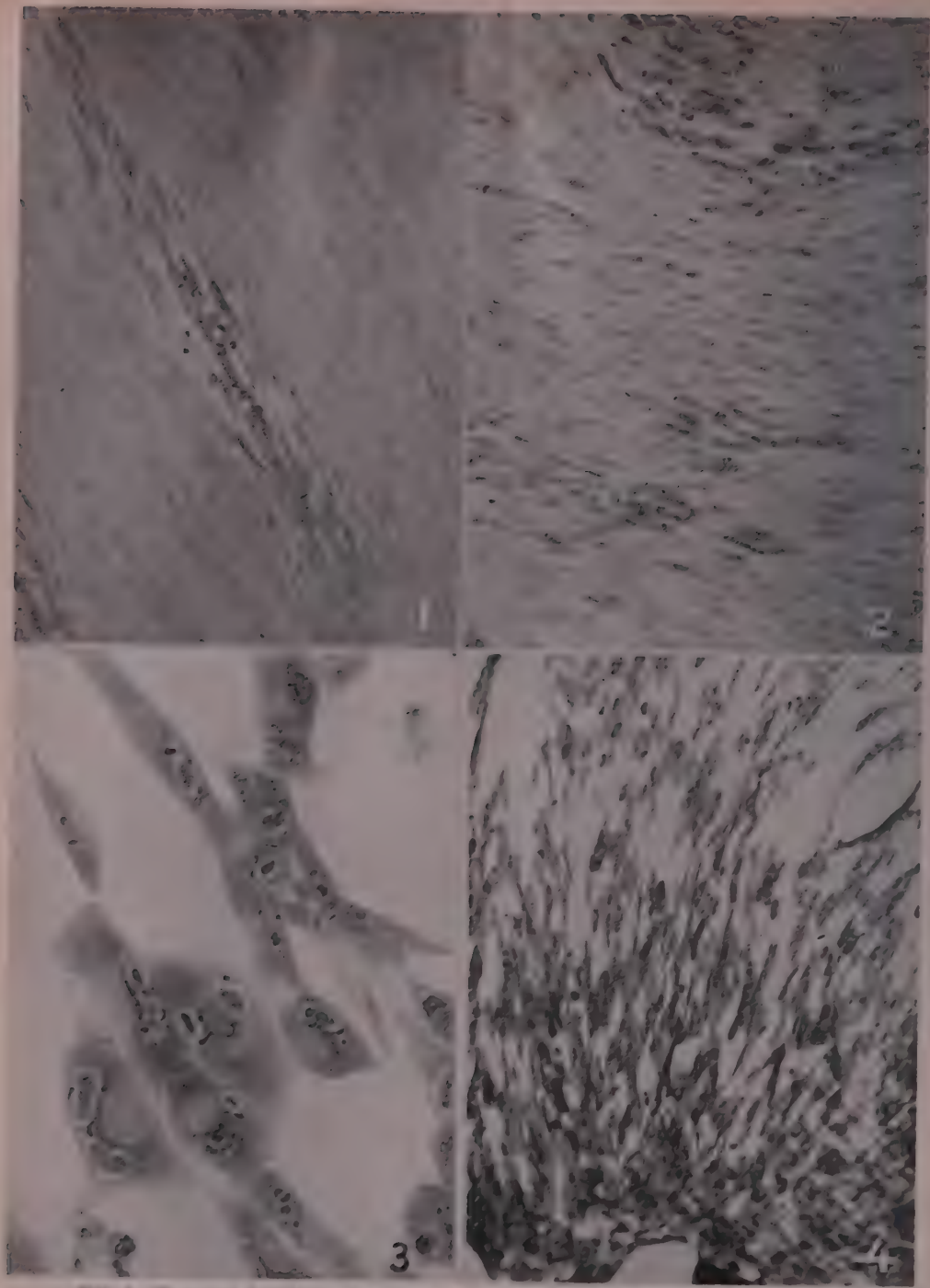

FIG. 1. Enlarged focal lesion in culture of normal uterine tissue, 29 days following inoculation of human salivary gland agent, original passage. Fixed and stained in culture tube, hematoxylin-eosin stain. × 90.

FIG. 2. Multiple small focal lesions from same culture as Fig. 1. × 90.

FIG. 3. High magnification of enlarged cell from a similar culture, showing intranuclear inclusions. Collodion embedded section, hematoxylin-eosin stain. × 515.

FIG. 4. Diffuse cytopathogenic changes in culture, 35 days following inoculation with human salivary gland agent, 4th passage. Intranuclear inclusions are seen in many of the enlarged cells. Collodion embedded section, hematoxylin-eosin stain. × 90.

serum, were used to replace the nutrient fluid on four 14-day-old cultures of human uterine tissue. An equal number of uninoculated control cultures of the same age were maintained under the same conditions as those to which the human material had been added, 1 for 29 days and the other 3 for 45 days. Eight other uninoculated cultures, implanted 4 days before isolation of the virus was attempted, served as additional controls. These were observed for periods varying from 30 to 45 days. In the isolation of the agent from renal tissue preparation of the inoculum from the tissue and the inoculation of 4 cultures were carried out in the same manner as in the isolation of the 1st agent from salivary gland tissue. Control uninoculated cultures were maintained under the same conditions as those inoculated with the renal tissue.

Cytopathologic Changes in Cultures. The early cytologic changes in the cultures consisted of small, round or oval foci in which the cells were enlarged, rounded or oval, and somewhat refractile in contrast to the normal fibroblasts (Fig. 1 and 2). On the 7th day following inoculation of the salivary gland material, 2 of these small foci were observed in one of the culture tubes, and on the 10th, 15th and 24th days, respectively, 2 or 3 similar small areas of altered cells were observed in the other 3 cultures. There was a continuous slow increase in the number and size of lesions in all tubes, followed by degeneration of cells in the centers of the lesions. The degenerating central cells became granular and disintegrated, leaving masses of dense refractile granules. One culture was fixed and stained on the 29th day after inoculation and the remaining cultures on the 56th day. Even at 56 days less than half of the cells of the cultures appeared to be affected.

Cytopathologic changes indistinguishable from those induced by the first agent appeared in the 4 cultures, inoculated with the kidney-material, on the 6th or 7th day following inoculation. The focal lesions increased in size and number somewhat more rapidly than those in cultures inoculated with the salivary gland-material. Thirty days after inoculation of the cultures many of the focal lesions had become confluent. In large areas of the cultures there were degenerating cells and dense granules from disintegrated cells, bordered by the altered large rounded or oval cells. Between these large areas apparently normal fibroblasts were still present. Cytologic changes of the same character occurred in subsequent serial subcultures of both viruses. No cytopathologic changes were observed in any of the control cultures.

In cultures of both viruses fixed and stained in the tubes large intranuclear inclusions were seen under low magnification (100x) in most of the enlarged rounded cells. Occasional cells had 2 or 3 nuclei, each containing an inclusion. The inclusions were amphophilic when stained with hematoxylin and eosin. In cultures embedded in collodion and observed under high magnification the inclusions were finely granular, and in some nuclei 2 or 3 separate masses were seen (Fig. 3). The inclusion was separated from the nuclear membrane by a distinct clear zone and the shape of the inclusion usually corresponded closely to that of the nucleus. Small masses of basophilic material were present in the nuclei, frequently in a marginal position. The nuclear changes closely resembled those seen in infected human tissues in cases of SGV infection and were indistinguishable from those induced in cultures of mouse tissue by the mouse salivary gland virus(9). In most of the affected cells there was a round eosinophilic area adjacent to the nucleus while the remainder of the cytoplasm was basophilic. The large basophilic cytoplasmic granules or inclusions that often occur in infected cells in the human disease were not observed.

Serial Passages. An attempt to subculture the first agent 23 days after inoculation was unsuccessful. However, successful transfers were made on the 30th, 37th and 40th days, and this virus has now been maintained through 29 serial passages over a period of 33 months (Table I, 12 serial passages). For the first 3 serial transfers, made at successive intervals of 37, 47 and 33 days, 2 ml of pooled fluids from preceding cultures were used to replace the entire amount of nutrient fluid on each new culture. The culture fluids were

TABLE I. Serial Propagation of a Cytopathogenic Agent Isolated from Salivary Gland of an Infant, in 12 Successive Passages.

Passage	Days in culture before transfer	ml of fluid transferred from previous cultures	Day cytologic changes noted	No. of cultures, cytologic changes / No. of cultures inoc.
1	0	.. ..	7, 10, 15, 24	4/4
2	37	2.0	7	2/2
3	47	2.0	5	2/2
4	33	2.0	2	2/2
5	30	.5	4	1/1
6	21	2.0	2	1/1
7	9	.2	2	2/2
8	14	.2	5	2/2
9	9	.2	3, 5	2/2
10	10	.2	5	3/3
11	21	.2	2	2/2
12	21	.2	2, 3	12/12

adjusted to pH 7.6 by the addition of 1 or more drops of 1.4% $NaHCO_3$, and 0.5 ml of 2% glucose per 2 ml of fluids was also added. In the successive serial passages cytopathologic changes appeared earlier, were more diffuse and spread more rapidly, involving the entire culture within 2 to 3 weeks (Fig. 4). The first 6 serial passages of the second virus were made at successive intervals of 30 to 56 days by replacing the entire medium on each new culture with 2 ml of pooled fluids from preceding cultures. In the first 4 serial subcultures the incubation period of the cytopathologic changes was 10 to 17 days and the focal lesions increased slowly in size and number. Thereafter the cytologic changes appeared earlier and, as with the first virus, were more diffuse and spread rapidly. The second virus has been carried through 12 serial passages.

At present the transfers of both viruses are made after 2 to 3 weeks of incubation by adding 0.2 ml of fluid from infected cultures to each new culture, and the cytopathologic changes appear in 2 to 5 days.

Infective Titre of Culture Fluid. The infective titre of the supernatant nutrient fluid from the 6th serial subculture of the first virus was studied. Cultures were inoculated with 0.2 ml of the undiluted fluid and with the same amount of each of 3 serial 10-fold dilutions. Two or 3 culture tubes were inoculated with each dilution. The characteristic cytologic changes occurred in all tubes, but the time at which these changes appeared increased progressively with the dilution of the inoculated infected fluid. Changes were observed in cultures inoculated with 0.2 ml of the undiluted fluid on the 2nd day but did not appear in 3 cultures inoculated with 0.2 ml of the 10^{-3} dilution until the 24th, 28th and 34th days.

Neutralization Tests. The source of virus used in neutralization tests was fluid from 3-week-old cultures of the virus which had shown cytopathologic lesions 2 or 3 days following inoculations. When 0.2 ml of fluid from such cultures was used as the inoculum, the incubation period of the cytopathologic change in several cultures rarely varied more than 2 days. Culture fluid was added to an equal amount of the serum to be tested. Nutrient fluid, in the amount of 1.6 ml, and 0.4 ml of the serum-virus mixtures was placed on each of 3 cultures. Horse serum-virus mixtures were used in the control cultures. The incubation period of the cytopathologic change and the progression of the lesions were noted. A serum was considered positive when the incubation period of the cytologic change in the test cultures was prolonged 5 days or more in comparison with the control cultures.

The first agent was neutralized by the sera from 2 infants who died in the newborn period of generalized SGV disease and by the serum of the mother of one of these infants. The latter serum was obtained 1 month after

birth of the infant. The sera of 2 infants, 1 day and 3½ months of age, who had no clinical evidence of SGV disease also neutralized this virus. Sera from 2 other infants, 6 months and 1 year of age, who had no evidence of SGV disease gave negative results. Three of the same sera were tested with the 2nd virus. The serum of one of the infants dying of SGV disease and the serum of the mother neutralized the 2nd virus, and a serum that failed to neutralize the 1st virus also failed to neutralize the 2nd virus.

Properties of the Viruses. Both viruses retained infectivity in tissues from autopsies after storage in dry ice, for 1 week and 2 months respectively. In some instances infective culture fluid has been preserved in dry ice for 6 months with no apparent loss of activity. However, preservation in dry ice has not been uniformly good. Both viruses pass through a Selas #015 filter (maximum pore radius, 1.4 microns) with no apparent loss of infectivity. In repeated experiments, infective fluids from cultures of either virus failed to induce cytopathologic changes in cultures of mouse embryonic tissue.

Groups of 8 suckling mice and of 8 mice, 4 weeks of age, were inoculated intraperitoneally and intracerebrally with infective fluid from cultures of each virus. The mice which had been inoculated intracerebrally remained well during an observation period of 30 days. Those which had been inoculated intraperitoneally showed no signs of illness and were killed 2 weeks following inoculation. No evidences of SGV infection or other disease were found at autopsy. In contrast to these results with the 2 strains of virus of human origin, the mouse SGV disease, as characterized by the specific intranuclear inclusions, was produced uniformly in mice from the same colony by intraperitoneal inoculation of infective fluid from cultures of the mouse SGV in homologous tissue(9).

One-tenth ml of infective fluid from cultures of each virus was placed on a scarified cornea of each of 2 rabbits. No lesions of the corneae or conjunctivae, or other evidence of illness developed. One-tenth ml of infective fluid from cultures of the 1st agent was also inoculated on the chorioallantoic membrane of each of seven 10-day embryonated hen's eggs. The membranes appeared normal when examined on the 3rd and 5th days following inoculation, and microscopic examination of the membranes revealed no pathologic changes.

Discussion. Two viruses, inducing identical cytopathologic changes in cultures of human fibroblasts, have been recovered; the first from the salivary gland of an infant in whom infection of the salivary glands with the SGV was established by microscopic examination and the second from the kidney of an infant dying of generalized SGV infection. In each instance the cytopathologic changes occurred in all of the 4 inoculated cultures but did not occur in any uninoculated control cultures which were observed over the same period. Therefore, it may be concluded that the cytopathogenic agents were derived from the human tissue. Neutralization tests were performed with only a small number of human sera but the results also indicate that the viruses are of human origin. Both viruses were neutralized by the serum of an infant dying of cytomegalic inclusion disease and by the serum of the mother of the infant. One human serum did not neutralize either virus. Moreover complement-fixing and neutralizing antibodies to our 1st virus have since been demonstrated in human sera by Rowe and associates(11). Their results strongly suggest that our 1st virus is closely related to or identical with 2 other recently isolated viruses; one recovered by Weller from a biopsy of the liver of an infant having clinical signs of cytomegalic inclusion disease and another isolated by Rowe and associates from adenoid tissue.

The large intranuclear inclusions produced in the cultures of human fibroblasts are like those which are pathognomonic of SGV disease, and the cytopathologic changes, including the intranuclear inclusions, are indistinguishable from those induced by the mouse SGV in cultures of mouse tissue(9). However, the 2 agents from human tissue are differentiated from the mouse SGV by their failure to induce cytopathologic changes in cultures of

mouse tissue or to produce SGV disease in mice.

The character and progression of the cytopathologic changes induced in cultures of human fibroblasts by the 2 viruses which we have isolated resemble somewhat those described by Weller(12) for the varicella virus. However, the behavior of these 2 viruses in cultures differs from that of the varicella virus in that they are present in the culture fluid and can be readily transferred by the fluid. In addition the conspicuous dense granules which occur in the centers of degenerating focal lesions are not described for the lesions produced by the varicella virus.

The restricted host pathogenicity of the 2 agents also differentiates them from the virus of herpes simplex, a common virus producing a large intranuclear inclusion which might be encountered in human tissue in the absence of recognizable infection.

Conclusions. A cytopathogenic virus inducing large intranuclear inclusions like those occurring in salivary gland virus disease has been isolated from the tissues of each of 2 infants. In both cases the virus has been propagated serially in cultures of human fibroblasts derived from uterine tissue. The distinctive cytopathogenic effects and the apparent species specificity of the 2 viruses, together with the isolation of each of the viruses from human tissue which contained the characteristic inclusions of salivary gland virus disease are substantial evidence that these viruses are strains of the human salivary gland virus.

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Sedimentation Behavior of Serum Lactic Dehydrogenase. (22499)

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(Introduced by Eugene Roberts).

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The elevation of serum lactic dehydrogenase (LDH) activity has been observed in many patients with neoplastic diseases(1,2). Since 2 components with LDH activity have been found in heart(3,4) it was of interest to ascertain whether the LDH activity found in the sera of apparently healthy individuals or in patients with neoplastic diseases is attributable to one or more moieties. A technic has been described(5) which permits the determination of the sedimentation pattern of an enzyme in crude preparations. This method was applied in the present investigation to the study of the sedimentation behavior of LDH activity in serum as a first step in determining the physical properties of the serum enzyme.

Materials and methods. For investigation of sedimentation behavior one part of serum was diluted with 4 parts of saline-phosphate buffer mixture to give final solution that was 0.145M with respect to sodium chloride and

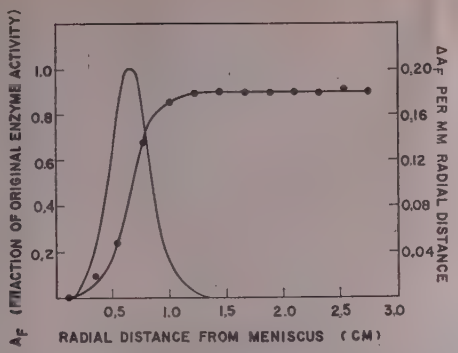

FIG. 1. Sedimentation diagram and increment curve obtained in a sampling experiment with serum lactic dehydrogenase from an apparently healthy individual (for details, see text). Sedimentation diagram, showing points determined experimentally, was plotted against left-hand ordinate and increment curve (no points) against right-hand ordinate.

0.025M with respect to phosphate (pH 7.4). For determination of sedimentation constant of LDH from erythrocytes heparinized plasma from a patient with myelogenous leukemia was centrifuged and the cells were washed with a volume of saline equivalent to original volume of plasma. Water, equivalent to original volume of plasma, was added to the washed cells, the mixture frozen and thawed 3 times, and centrifuged. The supernatant fluid was diluted 1:15 with saline-phosphate buffer mixture for investigation of sedimentation behavior. By means of a gradient pump (6), Lusteroid centrifuge tubes (1.2 x 5 cm) were filled with 4.50 ml of diluted serum or supernatant fluid from lysed erythrocytes in such a way that the fluid column within each tube contained a linear sucrose gradient ranging from 2% sucrose at bottom of tube to zero at the meniscus(5,7). Centrifugation was carried out in the SW-39 rotor of the Spinco Model E ultracentrifuge as previously described(7). At the rotational speed employed, 39,460 r.p.m., the distance of meniscus from axis of rotation was 6.09 cm. Following centrifugation, the tube contents were sampled(7), and 16 or 17 fractions consisting of successive levels of the fluid column from top to bottom were obtained. Immediately after sampling, all fractions together with original uncentrifuged solution were assayed for LDH activity by spectrophotometric method essentially as previously described(1). Reaction mixtures were incubated for 10 minutes for serum of high activity and 20 minutes for serum of low activity. Under these conditions rate of oxidation of dihydrodiphosphopyridine nucleotide (DPNH) was linear. Construction of sedimentation curves from sampling data required that the position of each point in the region of the boundaries be determined with considerable accuracy. Duplicate determinations of LDH activity, performed on each sample, gave identical results with only a few exceptions and these, on active specimens, never varied more than 1.7%. However, in the case of a few determinations on samples of very low activity the inherent error in the method gave an error as high as 5.5% in the results. Sedimentation diagrams (Fig. 1-3) were constructed from the sampling data of each experiment and translated into increment curves (Fig. 1-3) by methods previously described(5). Sedimentation constants ($s_{20,w}$) were calculated by means of standard equations(7,8). The density and viscosity of each solution were determined directly.

Results. Typical LDH sedimentation diagrams and increment curves obtained with serum are shown in Fig. 1 and 2. Fig. 3 illustrates the results obtained for LDH from a crude solution of lysed erythrocytes. The results of sampling experiments with LDH are

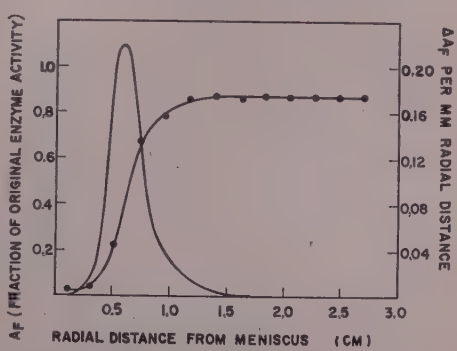

FIG. 2. Sedimentation diagram and increment curve obtained in a sampling experiment with serum lactic dehydrogenase from a patient with myelogenous leukemia (for details, see text). Sedimentation diagram and increment curve were plotted as in Fig. 1.

TABLE I. Determination of Sedimentation Constant of Lactic Dehydrogenase in Human Serum and Erythrocytes.

Centrifugation was carried out in the Spinco SW-39 rotor at 39,460 r.p.m. for 9360 sec. Solvent was .145M sodium chloride - .025M potassium phosphate, pH 7.4, containing sucrose concentration gradient ranging from zero at top of fluid column to 2% sucrose at bottom.

Source of serum	Approximate enzyme activity*	Avg temp., °C	$\Delta\chi$, cm†	$s_{20,w} \times 10^{13}$
Apparently healthy individual (non-fasting blood)	.16	23.1	.63	6.98
Idem (fasting blood), Fig. 1	.16	23.5	.67	7.20
Patient with myelogenous leukemia, Fig. 2	.77	23.2	.605	6.72
Idem (different patient)	1.28	24.8	.600	6.70
Lysed erythrocytes Fig. 3	38.0	25.0	.75	7.32

* Activity expressed as mg of DPNH oxidized/min./ml of original serum or ml of original packed erythrocytes assuming that decrease in optical density at 340 $m\mu$ of .200 is equivalent to oxidation of .1 mg of DPNH when determination is carried out as previously described(1).

† Radial distance from meniscus to peak of increment curve (see reference 5).

presented in Table I, together with the time, speed, and temperature under which the experiments were carried out. All sedimentation diagrams illustrated in Fig. 1, 2 and 3 exhibit a sharp boundary followed by a broad plateau region. The corresponding increment curves (Fig. 1, 2 and 3) consist of single, well defined peaks which are rendered somewhat asymmetric by an elevation of the leading limb.

Discussion. A major portion of the LDH activity of cancer serum was associated with a single component having a sedimentation constant ($s_{20,w}$) of 6.71 x 10^{-13} (average of 2 experiments). Normal serum and hemolyzed erythrocytes gave similar results with $s_{20,w}$ values of 7.09 x 10^{-13} (average of 2 experiments) and 7.32 x 10^{-13} (one experiment) respectively. Although there appears to be some difference between the above values, the variations are within the range of errors inherent in the centrifugation and assay methods. These values for $s_{20,w}$ are in good agreement with the following values in the literature: 7.0 x 10^{-13} reported for "heart" by Neilands(3), 6.36 x 10^{-13} and 6.48 x 10^{-13} for beef heart by Meister(4), and 7.39 x 10^{-13} for rat liver by Gibson(9). Neilands(3) and Meister(4) reported the existence of two lactic dehydrogenases from heart which differed in their electrophoretic mobility yet had the same sedimentation constant. It will, therefore, be necessary to employ other methods to determine conclusively whether the LDH activity of serum is attributable to one or more components.

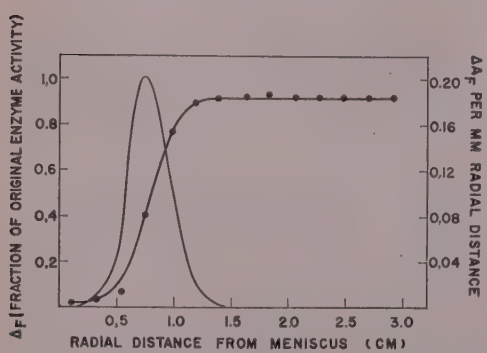

FIG. 3. Sedimentation diagram and increment curve obtained in a sampling experiment with hemolyzed erythrocytes from a patient with myelogenous leukemia (for details, see text). Sedimentation diagram and increment curve were plotted as in Fig. 1.

Summary. Lactic dehydrogenase from serum of an apparently healthy individual, sera from two patients with myelogenous leukemia, and lysed erythrocytes behaved as an essentially monodisperse compound, sedimenting at a rate which is in good agreement with that reported for the crystalline enzyme obtained from other sources. It will be necessary to employ other methods to determine conclusively whether serum LDH activity is attributable to one or more components.

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Vasopressor Effect of Synthetic 5-Hydroxytryptamine Creatinine Sulfate in Man.* (22500)

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Studies on hemostatic activity of 5-hydroxytryptamine creatinine sulfate (5-HT) indicated that administration of *small doses* of the drug into an antecubital vein caused often reflux of blood into the distal end of the plastic transfusion set, suggesting local elevation of venous pressure, while failing to affect systemic venous pressure(1). Further investigation was then undertaken on the fate of injected 5-HT(2) and on its effects on various circulatory districts.

Materials and methods. Fifty-two individuals, healthy or suffering from various diseases without elevation of venous pressure; one patient with ventricular septal defect and right to left shunt were studied. Seventy-nine experiments were performed. *Antecubital vein pressures* in one and or both arms were measured with saline manometers. On the injection side the pressure was obtained through the injection needle via a three-way stopcock during short, regular interruptions of the infusion. The system allowed separate infusions of drug or saline through the same needle. Brachial artery pressure was determined indirectly by means of aneroid sphygmomanometer. *5-HT*§ in crystalline form was dissolved in physiological saline to prepare solutions of various strength.

Results. (a) *Local venopressor effect of 5-HT and its relation to concentration of drug.* (Fig. 1). Saline solution was first injected intravenously at a speed of 3 ml/min. for 5 minutes, without effect on the local or contralateral arm vein pressure. By different orientation of the stopcock, 3 ml/min. of 5-HT solutions of various strength were then administered for 5 minutes in 3 different sets of experiments. Injection of saline was now resumed for 15 minutes. Dose of 0.15 γ/5′ 5-HT caused negligible venopressor effect; 1.5 γ/5′ induced significant elevation of local venous pressure, lasting at least 5 minutes after the end of the infusion; a 10-fold dose (15 γ/5′) determined more pronounced and sustained response. There was no *systemic* venopressor response when a total amount of 45 γ were injected in 15 subjects (15 γ/5).

(b) *Effect of repeated or continued intravenous administration of 5-HT on local venous pressure.* Repeated intravenous infusions of 5-HT (1.5 γ/5 minutes) were given; saline solution was administered for 15 minutes between injections (Fig. 2) Venopressor response to 5-HT was progressively more pronounced as injections were repeated. Local, severe pain prevented continuation of the procedure for more than 3 times in succession. It is then not known whether decrease in local venopressor effect might, as observed in dogs

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§ Kindly supplied by Dr. John Webb, Upjohn Co, Kalamazoo, Mich.

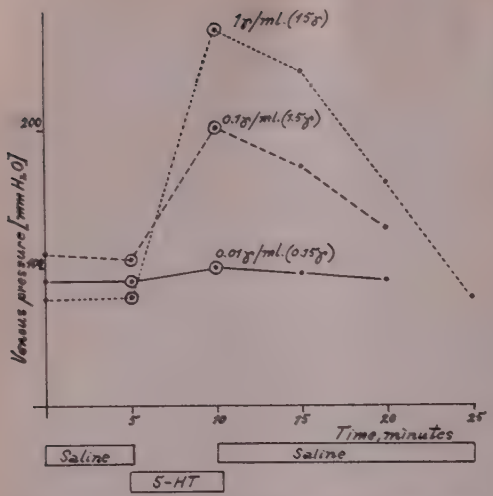

FIG. 1. Relation of concentration to local veno-pressor effect of 5-hydroxytryptamine creatinine sulfate (5-HT). (See text.)*

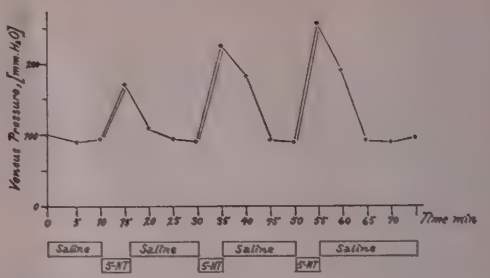

FIG. 2. Local veno-pressor effect of repeated administration of 5-hydroxytryptamine creatinine sulfate. (See text.)

* Total volume of 15 ml was inj. each 5 min. period at constant speed of 3 ml/min. Increasing concentrations of 5-HT induced more pronounced and sustained pressor effects. The curves traced were drawn from avg of 6 experiments.

(3), have followed further intermittent administration of 5-HT. Two individuals received 330 γ of 5-HT per minute in a volume of 1.66 ml/min. over a period of 30 minutes for a total dose of 10,000 γ. Speed of infusion was then doubled, the same total dose of 5-HT being injected within 15 minutes (Fig. 3). A maximum effect was quickly reached; further administration of the drug maintained, but did not significantly enhance, the veno-pressor effect. There was no elevation of systemic venous or arterial pressure.

(c) *Effect of rapid intravenous and intra-arterial administration of high doses of 5-HT.* Two typical studies are reported. A healthy, 16-year-old girl received *intravenously* 1,000 γ of 5-HT in 5 ml of saline in one minute time. Within 15 seconds, she experienced pain at site of injection, flushing sensation limited to face and neck for 2 to 3 minutes. Arterial

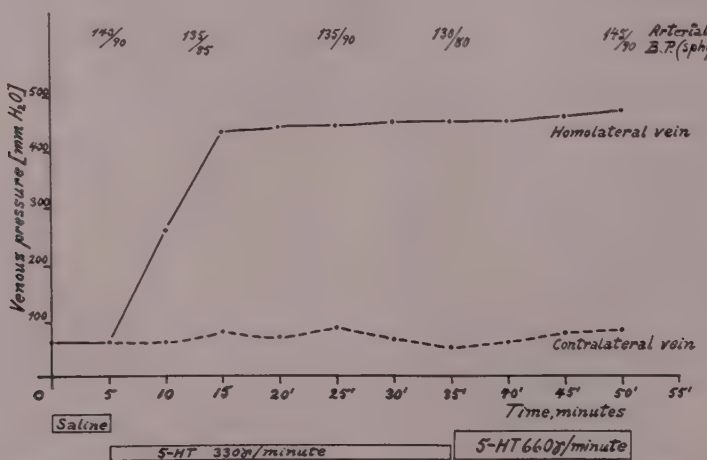

FIG. 3. Effect of continued administration of high doses of 5-hydroxytryptamine creatinine sulfate (5-HT) on pressure in homolateral and contralateral vein, and on arterial pressure (sphygmomanometric reading, values in mm Hg).* Graphic representation of an experiment G.M., a patient of Hodgkin's sarcoma in remission.

* A total of 10 mg of 5-HT was administered intravenously in 30 min.; then same amount was given in 15 min. by doubling speed of administration.

blood pressure rose from 130/64 to 150/110 mm Hg within 1 minute of the injection and returned to normal within 2 minutes. No rise of systemic venous pressure was noted. A 50-year-old male with generalized carcinomatosis received the same amount of 5-HT, *intraarterially*. Severe but fleeting pain followed immediately, radial pulse could not be felt for 5 minutes. After a short period of blanching, skin of volar and dorsal surface of the hand appeared flushed for longer than 30 minutes. Pressure rose from 117/80 to 130/86 mm Hg at the end of the injection; dropped to 100/60 mm Hg at the end of 5 minutes, returned to normal within 10 minutes. Pressure in the *homolateral vein* rose from 65 to 280 mm H_2O. Readings after 5′ 10′, 20′ and 25′ were 190, 155, 120 and 75 mm H_2O respectively. Again *there was no rise of pressure in the contralateral vein.*

(d) *Vasopressor effect of intravenous 5-HT in one patient with venous-arterial shunt.* A patient with ventricular septal defect and cyanosis was studied.‖ One ml of solution containing 200 γ of 5-HT was injected in one antecubital vein in one minute, a dose not followed by significant systemic effect in normal individuals. In this patient, however, injection induced immediate generalized "flushing" sensation and numbness. Arterial pressure rose from 110/80 to 125/100 mm Hg for about 5 minutes following the injection. Venous pressure recorded in the opposite arm remained unchanged.

Discussion... The drug 5-HT stimulates contraction of smooth muscle fibers(4), an attribute which may explain the local venopressor effect observed in our experiments. Vasopressor effect was obviously related to the concentration of 5-HT in each segment of the circulation at any given time. High doses given i.v. in very short time caused maximum local venopressor response and significant but only fleeting response in arterial pressure; administered over a longer period of time they induced only local venopressor effect. Fig. 1 and 2 indicate that the effect was specific rather than due to such non-specific phenomena as reflex venoconstriction due to pain or filling of the vein by solutions. Striking was the inability of 5-HT given intravenously at standard doses to affect systemic arterial pressure, the only exception being represented by the patient with venous-arterial shunt. Also, even large amounts of 5-HT given intravenously did not induce elevation of systemic venous pressure. Finally, intraarterial administration of 5-HT caused a sustained response in the homolateral, but not in the contralateral vein.

A number of speculative conclusions suggest themselves. Because of its fleeting action, 5-HT must be disposed of by the body quickly. *In vitro* experiments suggest that 5-HT may not be destroyed by aminoxidase (2). Partial by-passing of the pulmonary circulation (venous-arterial shunt) caused elevation of arterial blood pressure and generalized flushing at doses unable to do so in normal subjects. 5-HT may thus be retained within the lungs. Also, since the intraarterial injection of 5-HT was followed by sustained elevation of pressure in the homolateral vein, 5-HT might have been retained in and slowly released from the peripheral capillary bed. Storage in the pulmonary and peripheral capillary districts may then represent an important factor in the inactivation of injected 5-HT.

It has been shown that platelets absorb and carry 5-HT within the circulation(5). If platelets are trapped in the pulmonary as well as in the peripheral circulation, they may then retain and later slowly release absorbed 5-HT. Tissues also may possess a similar property.

Summary. 1. Intravenous injection of 0.3 γ/min. 5-hydroxytryptamine creatinine sulfate (5-HT) caused transitory elevation of local venous pressure; higher doses induced more marked elevation of local venous pressure and, when administered in short period of time, of arterial pressure. Elevation of systemic venous pressure was not obtained even with extremely high doses of 5-HT. 2. Intra-arterial administration of 5-HT induced sustained elevation of pressure in the homolateral veins (30 minutes or longer). 3. Elevation of arterial pressure and generalized

‖ This patient became available for study through courtesy of Dr. Richard H. Wright.

flushing followed injection into a patient with septal venous-arterial shunt of a dose of 5-HT unable to cause such effect in normal subjects. 4. It is postulated that injected 5-HT is quickly removed from the circulation, perhaps by platelets or by other cellular elements retained in capillary beds and released slowly at a later time.

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Leukemia Induction in Mice by Fast Neutron Irradiation.* (22501)

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This investigation was undertaken for the purpose of studying development of lymphoid, myeloid, and other types of leukemia in mice exposed to fast neutrons, as compared to X rays. Most cases of radiation-induced leukemia in human beings have resulted from exposure to gamma or X rays(1); however, in Japanese atomic bomb survivors the possible importance of neutron irradiation in the production of leukemia has been cited(2). Because neutrons have been observed to be, in general, relatively more effective than X or gamma rays in causing delayed radiation injuries in mice(3,4) and in man(5), determination of the relative biological effectiveness (RBE) of neutrons for leukemia induction is of practical, as well as fundamental importance.

Materials and methods. Male and female mice of the RF strain 8-16 weeks of age were exposed to fast neutrons in the 86-inch cyclotron of the Oak Ridge National Laboratory or to X rays. The neutrons, produced by the bombardment of an internal beryllium target with 22-Mev protons, had an average effective energy of approximately 1 Mev. The factors of X radiation were as follows: 250 kvp, 30 ma, TSD 93.7 cm, filtration 3 mm of Al (plus beryllium window), hvl 0.4 mm of Cu. Neutrons and X rays were administered to the whole body at an intensity of 65-115 rad/min in a single exposure or in two equal treatments separated by an interval of 2-8 days. Further details of the dosimetry and methods of exposure have been reported(6,7). After irradiation the mice were caged in groups of 8-10 in air-conditioned quarters, with Purina fox chow and drinking water available *ad libitum*. The animals were observed until natural death or were sacrificed *in extremis*. Postmortem examinations were performed on all mice.

Results. For a given dose of radiation the 30-day mortality was greater in neutron-exposed than in X-irradiated mice; in females the $LD_{50}/30$ days for a single exposure was approximately 335 rad of neutrons or 490 rad of X rays (RBE = 490/335 = 1.47)(7). With fractionation of the dose into two equal exposures, the $LD_{50}/30$ days for X rays and neutrons increased with the interval between treatments, being approximately 640, 710, 815, and 870 rad of X rays for intervals of 2, 4, 5, and 8 days, respectively(8). The acute injuries produced by neutron irradiation were indistinguishable from those caused by X rays except for acceleration of the 30-day mortality (the peak death rate occurred on the 5th day after single median-lethal neutron irradi-

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† Present address: Children's Cancer Research Foundation, Boston, Mass.

TABLE I. Incidence of Leukemia in RF Mice Exposed to Fast Neutrons and to X-Rays.

	No. of exposures	Time between expos., days	Neutrons: Dose, rad	Neutrons: Survival 30-day, %	Neutrons: Survival No. of mice*	Neutrons: Myeloid leukemia, %	Neutrons: Thymic lymphoma, %	Neutrons: Median survival, mo	X-rays: Dose, rad	X-rays: Survival 30-day, %	X-rays: Survival No. of mice*	X-rays: Myeloid leukemia, %	X-rays: Thymic lymphoma, %	X-rays: Median survival, mo
Male	0	0	0	100	80	3	4	21	0	100	250†	4	6	21
	1	0							119	100	46†	19	9	18
	1	0	110–165	100	28	43	7	12						
	1	0	170–195	100	25	32	12	12						
	1	0	205–230	100	41	44	5	12	335	100	65‡	29	17	16
	1	0	245–320	19–100	68	38	10	13	475	95	89†	11	17	14
Female	0	0	0	100	99	3	17	21	0	100	197†	0	16	21
	1	0							119	100	106†	19	14	17
	1	0	200–230	100	34	29	21	12	335	100	60‡	18	33	13
	1	0	240–335	63–100	28	25	28	13	475	95	89†	4	35	15
	2	2							440–535	85–100	35	43	37	9
	2	2	345–370	50– 55	18	11	11	12	580–675	40– 70	22	17	37	9
	2	5	320–360	85– 95	32	19	25	11	490–580	85–100	31	17	64	9
	2	5	380–385	70– 95	19	16	32	11	625–720	75– 85	31	19	52	10
	2	7–8	355–420	65– 85	33	11	35	12	580–720	65–100	27	24	66	7

* No. surviving to 5 mo of age, when first case of leukemia was encountered. † Data from previous exp.(10). ‡ Data from previous exp.(9).

ation and on the 9th day after exposure to X rays). After the first 30 days postirradiation, the death rate dropped nearly to control levels; 4 or 5 months later the survivors began to die of leukemia. Two major types of leukemia were induced, myeloid leukemia and thymic lymphoma. Most deaths from thymic lymphoma occurred in mice 6-15 months of age (mean 10 months); the incidence and rapidity of development increased with the dose of radiation. Thymic lymphoma was consistently more common in females than in males (Table I). Myeloid leukemia developed slightly later in life, the peak mortality from this disease occurring in mice 10-14 months old (mean 12 months); as with thymic lymphoma, its incidence and rapidity of development increased with the dose of radiation. Myeloid leukemia was more common in males than in females and was induced by relatively low doses of radiation (110 rad of neutrons or 119 rad of X rays). Thymic lymphomas were significantly more numerous in X-irradiated than in neutron-exposed mice, but the two types of radiations appeared, in general, to be of approximately equivalent over-all leukemogenic potency when compared at dose levels comparable in 30-day lethality (Table I). On fractionation of either neutron or X irradiation, the incidence of thymic lymphoid tumors was noticably elevated when the interval between exposures was prolonged to 5-8 days; however, the induction of myeloid leukemia was not correspondingly enhanced by fractionation. The length of the life span of the mice in the various treatment groups appeared to be correlated more closely with leukemia incidence than with dose or type of radiation or 30 day lethality.

Discussion. The induction of lymphoid and myeloid leukemia by radiation has been reported in RF mice exposed to X rays(9) and to thermal neutrons(10). The enhanced thymic lymphoma formation noted on fractionation of the irradiation at intervals of 5-8 days confirms that observed in C57 BL mice (11). It is noteworthy that the induction of myeloid leukemia was not also increased by fractionation of the dose.

A comparison of neutron- and X-irradiated

mice showed a consistently lower incidence of thymic lymphoma, along with an equal or greater frequency of myeloid leukemia, in the neutron-irradiated mice. A lower induction rate of lymphomas in CF_1 mice exposed to reactor fast neutrons, in comparison to the rate in mice treated with comparable doses of γ rays (27-32% in the neutron-irradiated vs 60% or more in the γ-irradiated mice) has also been noted by Henshaw *et al.*(3). These observations suggest that the RBE of fast neutrons for leukemia induction may vary with the type of leukemia induced; this effect deserves further investigation, since it may shed light on differences in the mechanisms of myeloid and lymphoid leukemia induction. Further studies may disclose a correlation between the earlier mortality of the neutron-exposed mice dying within 30 days postirradiation, which has been attributed to relatively more severe intestinal injury and less severe marrow damage(7), and the lower rate of induction of lymphomas by neutrons, as compared to X rays.

The relative over-all leukemia-inducing effectiveness of fast neutrons was not greatly different from their RBE for 30-day lethality if allowance is made for slight variation with different hematologic types of leukemia. This is in agreement with the observations of Henshaw *et al.*(3), who noted that on acute irradiation the RBE of reactor neutrons for 30-day lethality approximated that for reduction of longevity. It is also consistent with similar observations on the constancy of the RBE of thermal neutrons for acute lethality, leukemogenesis, and most other delayed effects in mice(10,12). Since, however, the RBE of neutrons for some types of delayed injury increased significantly with prolongation of the period of exposure(3-5), the RBE for leukemia induction may under conditions of protracted irradiation greatly exceed the value noted in the present experiment.

Summary. 1. Leukemias induced in mice of the RF strain by acute fast neutron irradiation in the cyclotron resembled in frequency and hematologic type those induced by X irradiation. 2. Neutrons and X rays appeared to have an over-all leukemia-inducing effectiveness comparable to their 30-day lethality effectiveness, although thymic lymphomas were significantly less frequent in neutron-exposed than in X-irradiated mice. 3. The induction of thymic lymphomas appeared to be enhanced by fractionation of either neutron or X irradiation at intervals of 5-8 days.

The authors are grateful to Mr. W. D. Gude, Mrs. E. S. Ledford, Miss R. J. Elliott, and Mrs. F. F. Wolff for technical assistance.

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Effect of Sodium Polyanhydromannuronic Acid Sulfate on Incidence of Ulcers in the Shay Rat.* (22502)

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It was shown by Babkin and Komarov(1) that chondroitin sulfate would reduce the proteolytic action of dog gastric juice. Later, Zaus and Fosdick(2) reported that chondroitin sulfate decreased the rate of digestion of gelatin sheets by commercial pepsin. More recently, Levey and Sheinfeld(3) noted that sulfated polysaccharides inhibit the proteolytic action of pepsin *in vitro*. Heparin, chondroitin sulfate and Paritol C, a clinical grade of sodium polyanhydromannuronic acid sulfate, were used in their studies. In addition, they found that chondroitin sulfate markedly reduced the incidence of ulcers in the Shay rat. In view of this more recent communication, we instituted a study in Shay rats to determine whether oral administration of sodium polyanhydromannuronic acid sulfate (Manuronate®) would reduce the incidence of gastric ulcers and whether sodium polyanhydromannuronic acid sulfate added to alumina gel (Amphojel®) would enhance the ulcer prevention properties of alumina gel.

Method. The method employed in this study is essentially that of Shay *et al.*(4). Male, Wistar rats ranging in weight from 120 to 170 g were fasted for 48 hours and the pylorus of each animal was ligated under ether anesthesia by the conventional technic. Immediately after ligation, the stomach was flushed with 4.0 ml saline and 2.0 ml of the proper medication was administered by stomach tube. No food or water was allowed during the remainder of the experiment. Nineteen hours after drug administration, each rat was sacrificed with ether, the stomach removed, opened and examined for ulcers under a dissecting microscope (7 X magnification). The protective action against ulcers, of sodium poylanhydromannuronic acid sulfate solution, various concentrations of alumina gel (expressed as Al_2O_3 equivalent) and mixtures of the two were observed.

Twenty to 25 animals were used at any one time. Five of these were saline controls and the remainder were used 5 or 10 to a group, usually 5, for the various test substances. The groups of animals for any one test substance were spaced as much as possible throughout the duration of the study, Two systems of ulcer gradation were used. The first consisted of 5 categories: (1) No ulcers; (2) $<$ 10 ulcers—none perforated; (3) $>$ 10 ulcers—none perforated; (4) Perforated ulcers—animal living; (5) Perforated ulcers—animal dead. These gradings for each drug at each dose level were plotted on a graph. The second system consisted of an arbitrary gradation of the severity of ulceration, taking into account all the above factors plus the distribution and size of ulcers. Seven categories were included here: 0 to 4+ ranging from negative to many large ulcers approaching perforation; 5+ perforated ulcers —animal living; 6+ perforated ulcers—animal dead. The sum of plus values divided by number of animals used is called the "ulcer index." On occasion, a rat was found dead which on careful examination showed no ulceration. It was assumed in these cases that death was due to other causes and the animal was discarded.

Results. The results obtained in this study are shown in Fig. 1. Alumina gel in a concentration equivalent to 0.25% Al_2O_3 resulted in a somewhat lesser degree of ulceration than seen in the saline control animals. The concentrations equivalent to 0.5 and 2.0% Al_2O_3 reduced ulcer incidence appreciably. Alumina gel in a concentration of 1.0% Al_2O_3, did not, in this series of experiments, fall in line with the usual reduction in ulcer formation. We have no explanation for this.

Sodium polyanhydromannuronic acid sulfate when added to 0.25% alumina gel in con-

* Presented at Am. Soc. for Pharmacol. and Exp. Therap. Meeting, Atlantic City, N. J., Apr. 16-20, 1956.

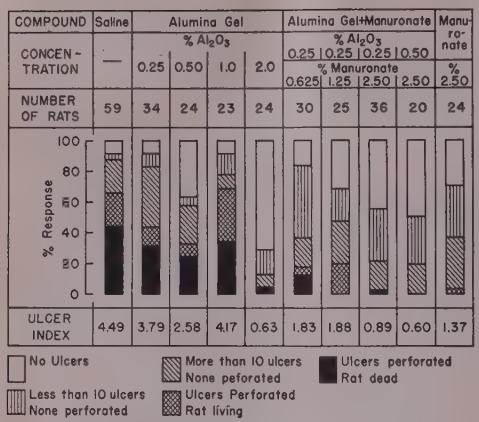

FIG. 1. Effect of alumina gel and Manuronate on incidence of ulcers in the Shay rat.

centrations of 0.625 and 1.25%, both greatly reduced ulcer incidence, the 2 showing approximately equal potency. An increase of sodium polyanhydromannuronic acid to 2.5% in 0.25% Al_2O_3 further decreased ulcer formation. Sodium polyanhydromannuronic acid sulfate, 2.5% in 0.5% alumina gel reduced ulcer formation still more.

A 2.5% solution of sodium polyanhydromannuronic acid sulfate without alumina gel, also reduced ulcer formation, the efficacy being approximately mid-way between 1.25 and 2.50% sodium polyanhydromannuronic acid sulfate in 0.25% alumina gel.

Summary. 1) A study has been made of the effects of sodium polyanhydromannuronic acid sulfate, alumina gel, and mixtures of the two, on incidence of ulcers in the Shay rat. 2) Each provided significant protection from ulcers and mixtures of the two reduced ulcer formation to a greater degree than either alone.

We wish to thank Mrs. Margot Gruenstein and Dr. Harry Shay for their advice concerning the method used.

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Relation of Sodium, Potassium, and Chloride Intake to Cortisone Action During Food Restriction. (22503)

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In a recent study(1), it was reported that the increase in renal size noted in rats on potassium low diets containing NaCl is not observed if the NaCl is replaced by $NaHCO_3$. It seemed desirable to extend these studies to include observations of food and water intake and to investigate the relation of cortisone to these functions on account of its importance for salt excretion. As on previous occasions, it seemed expedient to carry out the studies on rats whose weights were kept constant by restricted feeding.

Materials and methods. Male albino rats weighing approximately 100 g were kept individually in cages with wire bottoms and were provided with bottles suitable for the determination of water intake. They were weighed daily and supplied with enough food to keep their weight constant within ± 2 g. The requirements were eventually calculated as calories per gram body weight per week. Details of the procedures will be reported elsewhere(2). The basic experimental diet consisted of 30% test casein, 10% commer-

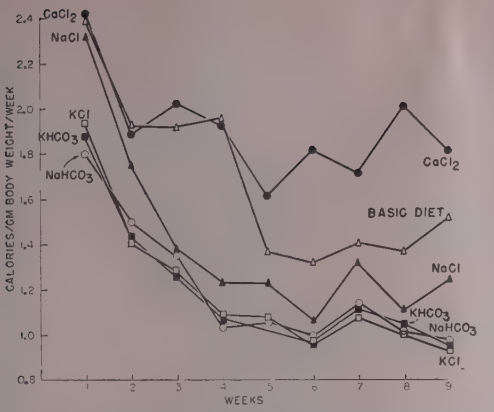

FIG. 1. Influence of various salt supplements on caloric requirements for weight maintenance, each point representing average for 8 male rats of about 100 g.

cial lard, 54% dextrose, 2% cellulose, liberal amounts of all vitamins,* and approximately 4% of a salt mixture containing the essential minerals except Na, K, and Cl. We estimate from the K and Na values known for test casein that the daily intake of the animals on the basic diet was about .2 mg of Na and 1 mg K.† The Cl intake may have amounted to 3 mg. When desired, this basic diet was supplemented with .92% NaCl, or equivalent amounts of $NaHCO_3$, KCl, or $KHCO_3$. Half equivalent amounts of $CaCl_2$ or NH_4Cl were used because animals fed these salts required roughly twice as much food for weight maintenance as those on the Na and K containing diets. Cortisone was fed in concentrations of 25 - 50 mg cortisone acetate per kilo of diet, which permitted a daily intake of .25 - .50 mg. In one experimental series, .5 mg cortisone acetate was injected subcutaneously in .1 cc of sesame oil 6 times weekly.

Results. In Fig. 1 are shown average caloric requirements for weight maintenance of groups on experimental diets with indicated salt supplements. Caloric requirements for weight maintenance of rats on a restricted intake of a "normal" diet decline sharply during the first 4 or 5 weeks and remain fairly constant thereafter(3). As can be seen from the figure, similar curves were obtained with all experimental diets. The decline was least pronounced for animals receiving $CaCl_2$, whose requirements were eventually twice as high as those of rats given Na or K salts. This finding was confirmed in 3 additional experiments. In 2 experiments with NH_4Cl, similarly high intakes were found. With the unsupplemented diet, food intake was usually not so high as with $CaCl_2$ or NH_4Cl but was much higher than with Na or K supplements. The caloric requirements of animals receiving $NaHCO_3$, $KHCO_3$, KCl, or a complete diet

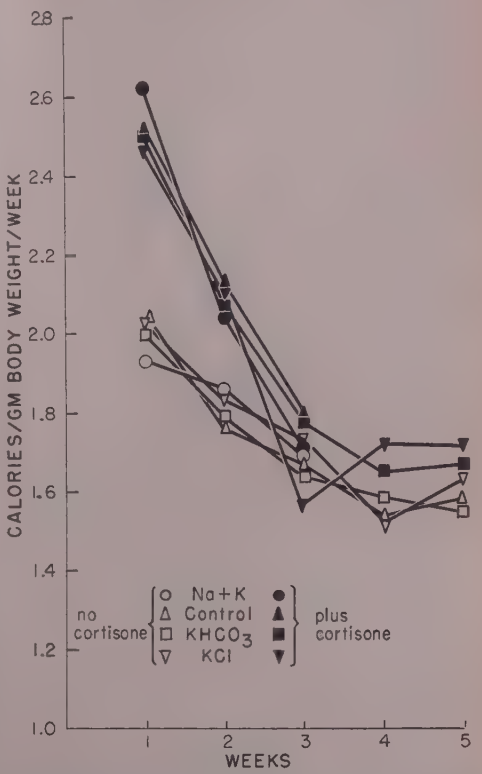

FIG. 2. Influence of cortisone acetate and K salt supplements on caloric requirements for weight maintenance, each point representing average for 8 male rats of 100 g. "Control" refers to group on complete diet.

* We are indebted to Dr. Leo Pirk, Hoffmann-La Roche, Nutley, N. J., who generously supplied us with most of the synthetic vitamins used. Vitamins were also received from Barnett Laboratories, Long Beach, Calif., Merck and Co., Rahway, N. J., and the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

† This was concluded from data kindly supplied by General Biochemicals, Chagrin Falls, O.

differed little from one another. In the experiment recorded in Fig. 1, requirements for the NaCl-containing diet were somewhat elevated, but this was true only 4 times in 8 series.

In another series similar to those just described, cortisone acetate was added to the diet. Fig. 2 shows that the initial caloric requirements of animals fed K salts and cortisone were much higher than those of animals given no cortisone. When animals on cortisone are permitted to eat freely, their food utilization is depressed(4). But, as Fig. 2 shows, low utilization (as reflected in high requirements) was observed only initially. The requirements decreased rapidly and eventually hardly differed from those of controls.

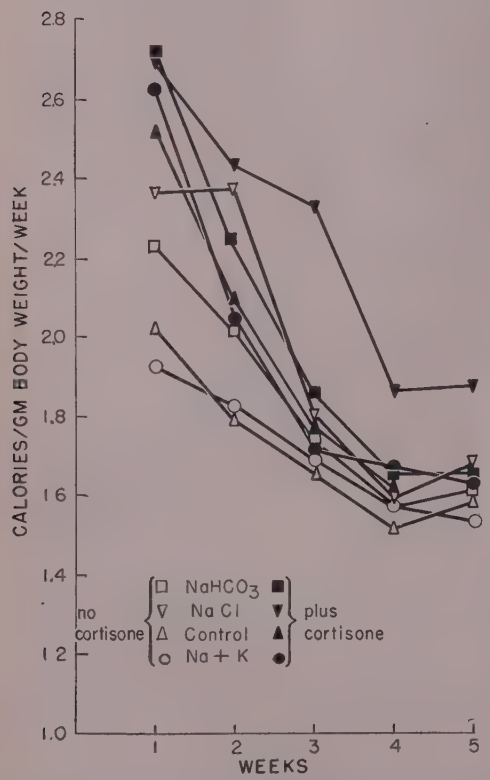

FIG. 3. Influence of cortisone acetate and Na salt supplements on caloric requirements for weight maintenance, each point representing average for 8 male rats of 100 g. "Control" refers to group on complete diet.

In Fig. 3 are given the results of feeding Na supplements. In general, the animals behaved like those with K supplements, the requirements of the cortisone groups being initially higher than those of their controls but declining to the same level. Only the animals receiving NaCl and cortisone ran persistently higher than all of the other groups, the differences being statistically significant in all 4 series.

With NH_4Cl supplements, requirements were just as high as with $CaCl_2$ (Fig. 1). Addition of cortisone to either the basic diet or that containing NH_4Cl made no difference; but when cortisone was fed along with $CaCl_2$, requirements were mildly yet significantly reduced in the 2 series carried out. Thus, cortisone had opposite actions in $CaCl_2$ and NaCl supplementations.

Fig. 4 gives the weekly individual water intakes. With $CaCl_2$, the consumption was 4 times as high as that of the controls on the complete diet. Addition of cortisone reduced the intake. The difference between the groups with and without cortisone was just on the borderline of statistical significance in this series but more pronounced in a second experiment. With NaCl, the water intake was initially twice as high as that of the controls on the complete diet but declined sharply. In two separate series with cortisone and NaCl, the intakes were eventually much higher than without cortisone.

Fig. 5 gives a log-log plot of the kidney weight against the body weight of animals kept on restricted food intake and given the various salt supplements with and without cortisone. The diagonal lines delimit the maximum spread for animals freely eating the complete diet. The renal weights of the controls and of the animals receiving KCl or $KHCO_3$ were within normal limits; administration of cortisone made no difference. With $CaCl_2$, the kidneys were twice as heavy as normal; addition of cortisone made for somewhat lower kidney weights in 2 experimental series. With NaCl supplements, kidneys were markedly heavier, as has been observed previously(5), and cortisone increased the weights further. In contrast, the kidney

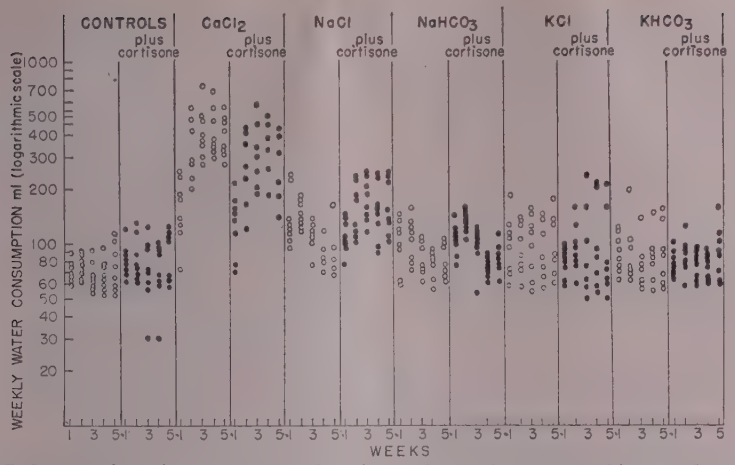

FIG. 4. Influence of cortisone acetate and various salt supplements on thirst, each point indicating weekly intake of one male rat of about 100 g kept at constant weight. "Control" refers to rats on a complete diet.

weights of rats fed $NaHCO_3$ were on the upper limit of the normal; cortisone led to mild increases.

The circumstance that cortisone exerts opposite effects on the kidney with NaCl and $CaCl_2$ is remarkable inasmuch as the histological lesions associated with the 2 supplements are identical.‡ This may deserve clinical consideration in the treatment of nephrosis.

When the relation of the adrenal weight to the body weight was studied, it became evident that, on the average, the adrenals were heaviest with NaCl and lightest with K salts. Cortisone and NaCl sometimes led to development of extremely heavy adrenals. With $NaHCO_3$, adrenals scarcely differed from those of controls on complete diet, and cortisone made no difference. With the basic diet or that supplemented with $CaCl_2$, adrenals were heavier than those of controls; cortisone reduced their size.

The liver weights of animals fed K salts without Na were lower than those of animals freely eating a complete diet. Cortisone was without effect. With $NaHCO_3$, livers were a little heavier and cortisone increased their weight slightly. With NaCl, livers differed little from those of the bicarbonate group, but cortisone substantially increased their size (after 6 weeks of food restriction, from roughly 3 to over 4 g in animals weighing about 100 g).

The thymic weights were reduced, as was to be expected; and various salts seemed to make no difference. The testicular weights were not remarkable.

Injections of cortisone did not change the results materially. It seemed, however, that the decline of caloric requirement for weight maintenance was, in the injected group, not quite so rapid as in the group given cortisone

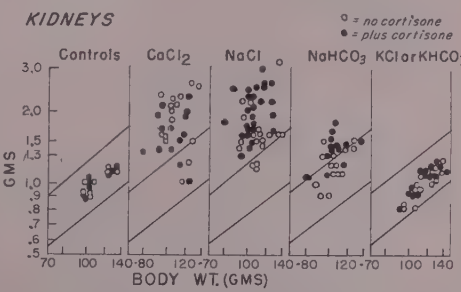

FIG. 5. Influence of cortisone acetate and various salt supplements on the kidney-body wt relation (log-log scale), each point representing one male rat of about 100 g kept at constant weight for about 6 wk. Diagonal lines delimit the spread for male rats freely eating a complete diet. "Controls" were kept at constant weight on the complete diet.

‡ The histological studies were carried out in cooperation with Dr. Herbert Stoerk of the Merck Institute for Therapeutic Research, Rahway, N. J.

in the diet. This was probably so because dietary cortisone intake of the latter animals declined as they ate less, whereas the daily dose of cortisone for injected animals remained constant.

Summary. 1. Caloric requirements, water intake, and organ weights were determined for rats whose weights had been kept constant by restricted feeding of diets low in Na, K, and/or Cl with and without cortisone acetate. 2. Whereas ordinarily requirements for weight maintenance decline sharply during the first 5 weeks and become constant thereafter, little if any decrease was noted without Na and K salts. With cortisone, requirements for all diets except those containing $CaCl_2$ or NH_4Cl were initially much higher than without cortisone but went down rapidly. However, with NaCl and cortisone, the requirements were higher than with K salts or $NaHCO_3$ and cortisone. On $CaCl_2$, the requirements were reduced by cortisone. 3. The water intake of animals receiving neither Na nor K was very high. Cortisone reduced the intake of the $CaCl_2$-supplemented rats. The opposite effect was noted with NaCl. 4. Organ weight determination reaffirmed that the increase in kidney weight noticed on NaCl-containing, K salt-low diets could be avoided by replacement of NaCl by $NaHCO_3$. The renal changes were therefore not the consequence of simple K deficiency. Cortisone enhanced renal enlargement in animals on K salt-low, NaCl-containing diets. 5. Cortisone acetate and NaCl reinforce, under certain conditions, some of each other's actions.

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Blood Gases in Cholera Patients and in Normal Subjects. (22504)

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Acute purging and vomiting in cholera produce marked reduction in plasma volume with concomitant increase in cell volume. This hemoconcentration affects the hemodynamics of circulation leading to alteration in the normal physiology of blood circulation. The only form of therapy which has stood the test of time as postulated by Rogers(1) is administration of solutions of sodium chloride to maintain the normal physiology of circulation. This form of treatment has been effective in many cases though some die in spite of the treatment, due to kidney failure, resulting in uremia. A reduction in alkalinity of blood was also reported(1,2). The cause of death in cholera is not definitely known. It might be due to acidosis. It might also be due to inadequate supply of oxygen to the tissues. In the present study, therefore, oxygen and carbon dioxide contents of the whole blood and the bicarbonate content of plasma in patients suffering from cholera and in normal persons were compared.

Methods. Patients admitted into the Nilratan Sircar Medical College Hospitals, Calcutta, with typical symptoms of cholera namely passage of rice-water stool, vomiting and collapse were selected. As a routine procedure stool was cultured and the cases whose stool showed the presence of cholera vibrio were included in the present report. Upon admission and before any treatment was rendered, blood was drawn from the antecubital vein with a dry syringe and delivered in a centrifuge tube under liquid paraffin. The tube was constricted in the middle forming a nar-

TABLE I. Oxygen and Carbon Dioxide Content of Whole Blood and Bicarbonate Content of Plasma of Normal Subjects and of Patients Suffering from Cholera.

Subjects	Whole blood		R.B.C.	Plasma bicarbonate	Packed cell vol
	O_2 vol, %	CO_2 vol, %	O_2 vol, %	vol, %	
Cholera (30)	11.3 ± .8	33.5 ± .9	24.2 ± 1.4	34.9 ± .4	57 ± 2
Normal (10)	10.2 ± .4	55.8 ± 1.5	22.2 ± .8	60.0 ± 1.5	46 ± 1
Difference of means	1.1	22.3	2.0	25.1	11
Stand. error of difference	.897	1.719	1.667	1.5	2.423
t	1.3	13.0*	1.2	16.7*	4.5*

* These values of *t* are highly significant.
Figures in parentheses indicate No. of subjects.

row neck so that the needle of the syringe could just pass through it. The centrifuge tube contained crystals of a mixture of ammonium and potassium oxalates so that when mixed with blood it was isotonic. As soon as the blood was collected a portion was taken in a syringe under liquid paraffin and analysed for oxygen and carbon dioxide contents by Van Slyke's manometric method(3). Packed cell volume was determined by centrifuging the blood in a Wintrobe tube. The rest of the blood was centrifuged, plasma separated and bicarbonate in plasma was determined by the method of Van Slyke *et al.*(4). The normal persons selected were post graduate and research students of the department of physiology of the Presidency College, Calcutta. The results are given in Table I.

Results. The oxygen content of the whole blood was 11.3% in cholera cases and 10.2% in normal subjects. The carbon dioxide content of the whole blood was 32.5 volume % in cholera cases and 55.8% in normal subjects. The plasma bicarbonate contents of cholera patients and normal subjects were respectively 34.9 and 60.0 volume %. The packed cell volume was 57% in cholera cases as compared to 46% in normal subjects.

Discussion. The packed cell volume increased significantly in cases of cholera due mainly to the loss of plasma. The carbon dioxide contents of the whole blood diminished to a marked degree in cholera cases. Plasma bicarbonate level also decreased significantly in patients suffering from cholera. The diminished alkali reserve of blood indicates a condition of acidemia in cholera. This is in confirmation of the findings of other workers. The oxygen content of the whole blood and also of the red blood cells, in patients suffering from cholera, did not differ from the corresponding values in normal persons. This indicated that cholera patients did not suffer from deficient oxygenation of the tissues and as such death in cholera is not due to anoxemia.

Summary. Packed cell volume, oxygen and carbon dioxide content of the whole blood and bicarbonate of the plasma were determined in 30 cholera patients and in 10 normal subjects. While there was no change in the oxygen content of the whole blood in patients suffering from cholera, the patients had a low plasma level of bicarbonate. Cholera patients suffer from acidemia and not from anoxemia.

We are indebted to Dr. A. K. Datta Gupta, principal of the Nilratan Sircar Medical College Hospitals, for the facilities provided.

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Inhibition of Isoniazid Acetylation *in vitro* and *in vivo*.* (22505)

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Previous studies have shown that isoniazid (INH) and sulfanilamide are acetylated by the same enzymic system, and that competitive inhibitors of sulfanilamide acetylation are inhibitors, also, of INH acetylation in pigeon liver extracts(1,2). The present report concerns the inhibition of INH acetylation *in vitro*, and its application *in vivo* as exemplified by the effect of p-aminosalicylic acid (PAS), and of sulfanilamide on the INH levels in the blood plasma of rabbits. A colorimetric method is described for the determination of free INH in the presence of aromatic amines.

Materials and methods. Pigeon liver extract was prepared as previously described(2). One ml of the extract was added to each reaction tube after all other additions had been made. In addition to pigeon liver extract all tubes contained the following substances, in final concentration, to a total volume of 3 ml: potassium phosphate buffer, pH 7.4, 0.02M; potassium acetate 0.02M; potassium citrate 0.02M; sodium adenosine triphosphate 0.004M; INH, 329 μg (8×10^{-4}M); acetylation inhibitors as indicated in Table I. The tubes were incubated for 30 min. at 37°C in the presence of air. 0.2 ml aliquots of the appropriately diluted incubation mixture were taken for the determination of free INH. The extent of acetylation was taken to be the difference between the free INH content of the incubated tubes and that of the zero-time control. In the absence of an acetylation inhibitor, approximately 50% of the added INH was acetylated during the 30 minute incubation period. Female albino rabbits were used for the *in vivo* experiments. They were maintained on a constant diet of Purina Rabbit Chow (100 g per day) containing all necessary vitamins and dietary factors, and water *ad libitum*. All drugs were given by mouth as a solution or suspension in tap water. A modification of the chlorodinitrobenzene method of Scott(3) was used for the measurement of INH in tissue extracts and plasma. Acetyl-INH and isonicotinic acid do not react with this reagent, and none of the acetylation inhibitors used in this investigation interfered with the determination of INH. Thus free INH can be selectively measured in the presence of its acetyl derivative and of aromatic amines and amides.

Procedure for tissue extracts. 0.2 ml of extract containing 10 to 20 μg of INH is pipetted into a 20 x 170 mm Pyrex test tube containing 200 mg of dry powdered borax. Ten ml of a 2.5% (W/V) solution of chlorodinitrobenzene in dehydrated ethanol is added. After mixing, the tube is placed in a boiling water bath for 20 minutes to allow evaporation of the alcohol. The tube is then cooled for 1 minute in ice-water, made up to 10 ml volume with methanol, agitated, and centrifuged at 3000 r.p.m. for 2 minutes. The optical density of the supernatant is measured at 530 mμ against a blank prepared in the same way but omitting INH. The amount of INH present in each sample is ascertained from a calibration curve prepared from appropriate standards run with each set of determinations. The optical density measured at 530 mμ is a linear function of INH concentration within the range 0 to 20 μg and reproducibility is $\pm$ 3% at the 15 μg level. *Procedure for blood plasma*—0.5 ml of clear plasma is pipetted into a centrifuge tube containing 6 ml of dehydrated ethanol. After mixing and standing for 5 minutes the tube is centrifuged. Five ml of the clear supernatant is transferred to a 20 x 170 mm Pyrex test tube containing 200 mg of powdered borax. Five ml of a 5% (W/V) solution of chlorodinitrobenzene in ethanol is added, and the treatment continued as described above for extracts. Appropriate standards containing known amounts of INH added to plasma are run with each set of determinations.

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TABLE I. Inhibition of Isoniazid Acetylation in Pigeon Liver Extracts.

Inhibitor	Inhibitor conc., M/l × 10^{-4}	Inhibition, %
2-Hydroxybenzamide (salicylamide)	10	36
2-Chlorobenzamide	10	0
4-Hydroxybenzamide	10	10
2-Hydroxy-5-bromobenzamide (5-bromosalicylamide)	1.3	52
2,5-Dihydroxybenzamide (gentisic acid amide)	2.5	54
3,4,5-Trihydroxybenzamide (gallic acid amide)	8	29
2-Hydroxy-3-methylbenzamide (o-cresotamide)	10	36
2-Hydroxy-4-aminobenzamide (p-aminosalicylamide)	10	86
2-Hydroxy-4-aminobenzoic acid (PAS)	10	49
4-Aminobenzoic acid (PABA)	10	41
4-Aminophenylacetic acid	20	36
0-Hydroxybenzal isonicotinylhydrazone (Nupasal-213)*	5	70
1-Hydrazinophthalazine (Apresoline)†	10	53
Sulfanilamide	9	30
N^1, N^1-Diethylsulfanilamide	2	74
N^1-Acetylsulfanilamide (sulfacetamide)	8	13
Sulfamethylthiodiazole	8	54
Sulfamethazine	8	16
6-Aminonicotinamide	10	50
4-Amino-5-imidazolecarboxamide	20	31

* Provided through the courtesy of Smith and Nephew Research Ltd., Ware, Herts., England.

† Provided through the courtesy of Dr. W. Murphy, Ciba, Montreal.

Experimental conditions are described under "Materials and methods."

Acids were brought to pH 7.4 with NaOH.

Results. The inhibitory activity of each of the 20 compounds listed in Table I is dependent upon the presence of an amino, amido, or hydrazino group in the molecule. The contribution to inhibitory potency made by other substituent groups has been discussed previously(2) and will be mentioned only briefly here. The strongly activating effect of the 5-bromo and 5-hydroxy groups of 5-bromosalicylamide and gentisamide, respectively, appears to be mediated through the 2-hydroxy group, since benzamide and 5-bromobenzamide (not shown in Table I) have no activity at 10^{-3}M. This is also indicated by the fact that gallamide which possesses the 5-hydroxy group, but lacks the 2-hydroxy, exerts only about one-sixth the inhibition of gentisamide. p-Aminosalicylamide appears to have the combined effect of PAS and salicylamide.

N^1, N^1-diethylsulfanilamide was the most effective sulfonamide tested. It is surprising that sulfamethylthiodiazole, which is poorly acetylated *in vitro* and *in vivo* proved to be somewhat more effective than sulfanilamide, which has a high rate of acetylation. The inhibition by sulfamethylthiodiazole may be due to blockage of active centers of the acetylating enzyme.

The primary amino group of 6-aminonicotinamide can be acetylated, thus accounting for its inhibitory effect. When this compound was fed to rabbits, 6-acetylaminonicotinamide was detected chromatographically as a urinary excretion product (unpublished results).

As previously mentioned(1), PAS, administered concurrently with INH to rabbits, produced a marked increase in the free INH plasma level. This is illustrated by the experimental results shown in Table II. The free INH concentration of plasma was measured 1, 2, and 4 hours after a single dose of INH, as indicated in the table. After a 48 hour interval, INH and PAS sodium were administered concurrently and plasma INH again measured. (Twenty-four hours after the administration of a single dose of INH there was no detectable INH in the plasma and hence no carry-over from one experiment to the next.) In the presence of PAS, the INH plasma levels after 4 hours were more than doubled in each individual case. This effect of PAS on INH plasma levels has been recently corroborated by Mandel *et al.*(4), who found that concurrent administration of PAS with INH resulted in detectable eleva-

TABLE II. Effect of PAS (Sodium) on Plasma Levels of Free INH in 3 Rabbits.

Wt, kg	Drug dose (mg/kg) INH	PAS (sodium)	Plasma levels of free INH (mg/100 ml) at 1 hr	2 hr	4 hr
6	50	—	4.8	2.4	1.4
	50	500	6.3	5.2	2.9
6	50	—	3.1	2.1	1.1
	50	500	4.9	3.4	2.4
3	50	—	2.24	—	.64
	50	500	2.86	—	1.86

TABLE III. Effect of Sulfanilamide (SAM) on Plasma Levels of INH in Rabbits after a Single Oral Dose.

Rabbit No.	Drug dose (mg/kg) INH	SAM	Plasma level of free INH (mg/100 ml) At 1 hr	At 5 hr
1	50	—	3.3	.65
	50	150	3.8	1.72
2	50	—	3.9	.57
	50	150	3.95	1.70

tion of the active INH level in the blood serum of tuberculous patients. The effect of sulfanilamide on the INH level of plasma is shown in Table III. The greater effectiveness of sulfanilamide as compared with PAS can partly be attributed to the higher excretion rate of the latter.

Discussion. When INH is administered orally to humans, from 50 to 90% of it is eliminated in the urine as 1-isonicotinyl-2-acetylhydrazine(5,6). Acetyl-INH has negligible antitubercular effect(7) and is rapidly excreted. The increased plasma levels of free INH attained by the concurrent administration of INH and PAS or sulfanilamide to rabbits are undoubtedly due to the effect of the latter compounds on INH acetylation. Mandel *et al.* have suggested that the increased therapeutic effect of INH plus PAS over INH alone may be due in part to the enhanced blood levels of free INH resulting from the inhibition of INH acetylation by PAS. To some extent, a somewhat similar effect may be the explanation for the more rapid and profound therapeutic effect observed by Selikoff, *et al.*(8) when the total daily dose of INH was divided into 3 or 4 doses. Thus, there are indications that the maintenance of therapeutic blood concentrations of INH may be of importance in tuberculosis therapy, views to the contrary not withstanding(9-11). As is known to be the case with sulfonamides, it is probable that the mean blood level of free INH, and not the total amount of INH absorbed is the important factor in the effectiveness of INH therapy.

The possibility of increasing the therapeutic effectiveness of a given dose of INH by the concurrent administration of a non-toxic acetylation inhibitor should be explored. For this purpose PAS is not the ideal inhibitor of acetylation, since it is acetylated only to the extent of 40 to 50% in humans and is excreted largely as a glycine conjugate(12). A sulfonamide retaining adequate solubility in the acetyl form would appear to show greater promise, as would the amide of PAS and other compounds listed in Table I. For that matter, PAS could still be retained in the composition for its property of delaying the development of bacterial resistance to INH.

Summary. The acetylation of INH by pigeon liver extracts was found to be inhibited by various amino, amido, and hydrazino compounds. PAS, administered to rabbits in conjunction with INH, produced a marked increase in the plasma level of free INH. A similar effect was obtained with sulfanilamide. A colorimetric method for the determination of free INH in the presence of aromatic amines is described.

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Studies on Distribution of Dicumarol. (22506)

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It has been shown that under certain experimental conditions between 60 and 80% of the dicumarol (3, 3'-methylenebis-(4-hydroxycoumarin)) in liver from dicumarol-treated rats is associated with the supernatant that remains after centrifugation of the mitochondria(1). As vit. K_1 effectively antagonizes dicumarol it seemed of interest to see whether the vitamin alters either the intracellular localization of the drug or its total concentration in liver or in blood. We have also measured the distribution of dicumarol in several tissues of the rat and chick.

Methods. Chicks and rats used in the experiment received a commercial chicken ration(2). The animals were fed dicumarol diluted in fructose for 2 successive days. Rats received 0.1 mg dicumarol per g body weight; chicks 0.7 mg per g body weight since they are relatively resistant to lower doses(3). Vit. K_1 was given at a level of 0.1 mg per g body weight in the form of a transparent colloidal solution in water(2). Forty-eight hours after the first treatment, prothrombin times were determined by the method of Dam and co-workers(4) on blood obtained from the jugular vein. The normal prothrombin times for chicks and rats are about 18-21 seconds by this method. The rats and chicks were then killed by decapitation (the rats after a blow on the head), and the tissues were homogenized in isotonic sucrose with Teflon homogenizers. Cellular fractionation was carried out as described by Schneider and Hogeboom (5), except that after separation of the mitochondria the remaining supernatant was not further fractionated. Dicumarol was determined by the method of Weiner and associates(6). Preliminary experiments showed that the apparent amount of "dicumarol" in livers of untreated chicks varied from 0.8 to 1.9 μg per g liver; rat liver had no blank dicumarol. The recovery of dicumarol added to liver homogenates from normal animals varied from 84 to 103%.

Results. The representative experiments shown in Table I demonstrate that vit. K_1, while markedly reducing prothrombin time, failed to influence concentration of dicumarol in either whole liver or its subcellular fractions. Dicumarol levels in chick liver were higher than in rat liver, which can probably be attributed to the higher amount administered to the chick. Mitochondria and supernatant fractions of chick and rat liver contained about the same amount of dicumarol; the nuclear fraction of chicken liver contained 2-3 times the quantity of dicumarol found in the same fraction of rat liver. Undoubtedly part of the dicumarol found in the supernatant represents intravascular dicumarol. Livers from chicks given dicumarol in doses of 0.1 mg per g body weight on two consecutive days and killed 24 hours after the last dose contained only insignificant amounts of dicumarol.

In Table II, it may be seen that vit. K_1 did not influence blood levels of dicumarol although it prevented prolongation of the prothrombin times by dicumarol.

Table III shows that liver, blood and kidney contain the highest concentration of dicumarol, as has been observed previously for dicumarol(6,7) and other coumarin anticoagulants(7-9). Part of the drug that appears to be in tissues is actually in blood: in one experiment, perfusing the liver decreased its dicumarol concentration from 24 to 15 μg per g.

Discussion. After one injection of the dose of vit. K_1 used in these experiments the liver contained about 50% of the injected amount (10,11). The deposition is assumed to take place mainly in reticulo-endothelial tissue (10). The level of vit. K_1 used (0.1 mg per g body weight) corresponds to about 1200 times the dose that would have produced normal prothrombin time in 20 hours when given

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TABLE I. Intracellular Distribution of Dicumarol.

Species	Body wt, g	Treatment* 0 hr	24 hr	36 hr	48 hr	Prothrombin time	μg dicumarol/g liver: Whole homogenate	Nuclei	Mitochondria	Supernatant
Chick	344	D K_1^{iv}	D	K_1^{iv}	k	21″	72	31	9	28
	356	D K_1^{o}	D K_1^{o}	K_1^{o}	k	2′ 10″	74	31	9	29
	346	D	D		k	10′ 00″	78	33	8	29
Rat	287	d K_1^{iv}	d K_1^{iv}		k	18″	56	14	9	34
	298	d	d		k	3′ 12″	44	9	6	32

* D = Dicumarol, .7 mg/g body wt, orally. d = Dicumarol, .1 mg/g body wt, orally. K_1^{iv} = Vit. K_1, .1 mg/g body wt, intravenously. K_1^{o} = Vit. K_1, .1 mg/g body wt, orally. k = killed.

orally to vit. K deficient chicks(4). We have given 2 such injections and yet, while preventing dicumarol-induced hypoprothrombinemia, the vitamin did not change either the liver content of dicumarol or its distribution among the liver fractions. Conversely, other experiments suggest that coumarin-anticoagulants may cause a prolonged prothrombin time even when the liver contains large stores of vit. K_1

TABLE II. Blood Levels of Dicumarol.

Chick body wt, g	Treatment* 0 hr	24 hr	48 hr	Prothrombin time	μg dicumarol/ml blood
359	D K_1^{iv}	D K_1^{iv}	k	18″	59
383	D K_1^{iv}	D K_1^{iv}	k	18″	62
390	D	D	k	1′ 43″	30
387	D	D	k	2′ 04″	53

* D = Dicumarol, .7 mg/g body wt, orally. K_1^{iv} = Vit. K_1, .1 mg/g body wt, intravenously. k = killed.

(11). Correction of a dicumarol-induced hypoprothrombinemia by vit. K_1 does not appear to depend on gross displacement of the drug from either whole liver or its subcellular fractions. Nor does the vitamin appear to affect the blood levels or metabolism of the drug.

Lee and co-workers(12) have shown that injection of vit. K in the form of 2-methyl-1, 4-naphthohydroquinone phosphoric acid ester favored more rapid displacement of dicumarol from the livers of rabbits and mice. As they administered dicumarol intravenously and determined dicumarol at shorter intervals, the results cannot be directly compared. Jaques (13-15) has emphasized that there exists a correlation between the time dicumarol remains in the liver and the duration of hypoprothrombinemia. Others(16-18) have noted that, in general, plasma levels of dicumarol and related drugs are correlated with duration of the prothrombin response. Our experiments indicate that dicumarol levels in both liver and blood may remain elevated at the same time that prothrombin times become normal.

Summary. Vit. K_1 given intravenously in massive doses to dicumarol-treated rats and chicks did not influence the concentration of dicumarol in whole liver, the subcellular fractions (nuclei, mitochondria and supernatant) of liver, or whole blood, while the usual dicumarol-induced hypoprothrombinemia was prevented. The tissue distribution of di-

TABLE III. Tissue Distribution of Dicumarol.

Species	Body wt, g	Prothrombin time	μg dicumarol/g or ml of: Liver	Kidney	Lung	Spleen	Brain	Heart	Skeletal muscle	Blood	Plasma
Chick*	365	4′ 42″	55	48	21	36	16	32	7.2	49	73
	340	8′ 32″	65		16	30	13	28	7.8	42	68
Rat*	266	2′ 25″	24	15	7.9	2.3	1.8	5.3	2.2	11	
	286	4′ 38″	18	9.8	6.8	.8	1.4	2.5	1.9		
	244	4′ 40″	45	21	20	1.4	5.6	14	5.7	35	
	286	5′ 50″	40	18	19	1.7	2.3	12	2.4	23	

* Chicks and rats were treated with dicumarol given in doses of 0.7 mg and 0.1 mg/g body wt, respectively, on 2 consecutive days and killed 24 hr after the last dose.

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Increase in Induced Pulmonary Tumors in Mice Associated with Exposure To High Concentration of Oxygen. (22507)

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Reports on the effect of concentration of oxygen upon mutation rate have been of considerable interest to those investigating relationships between mutagenesis and carcinogenesis. The original report was that of Thoday and Read(1) who observed that bean root cells X-irradiated in nitrogen contained a lower proportion of chromosomal aberrations than those irradiated in oxygen. Subsequent work(2-4) has shown that the concentration of oxygen affects the rate of gene mutations as well as that of chromosomal changes, and has also shown that the effect is not limited to irradiated cells. Increased interest in possible relationships of these observations to carcinogenesis arose when Plaine and Glass(3) reported an increase in occurrence of melanotic tumors in *Drosophila* larvae irradiated in the presence of increased concentrations of oxygen. Recently Plaine (5) has reported an increase in the melanotic tumors with increase in concentration of oxygen without radiation.

Comparison of these melanotic tumors in *Drosophila* with neoplasms in mammals morphologically is limited, but genetically similarities between the two exist. This fact together with the observations that many agents known to increase mutation rate in lower organisms are carcinogenic when tested in mice made it highly desirable to test the concentration of oxygen on the occurrence of neoplasms in mice. The fact that the effects of oxygen in the lower organisms was not limited to irradiated cells increased the possibilities for a study of the effect of concentration of oxygen on carcinogenesis. The purpose of this report is to record an increase in occurrence of pulmonary tumors in mice exposed to a high concentration of oxygen.

Materials and methods. Pulmonary tumors in the genetically highly susceptible strain A

mice were chosen for the study since in our experience they have provided a very delicate test for effects of agents upon carcinogenesis. The intravenous injection of the carcinogen dibenz[*a*,*h*]anthracene was added to the procedure in order to produce multiple tumors so that any effect could be measured on a quantitative basis and also in order to cut down the length of time necessary to keep the mice before making the observation. From former dose-response results(6) a dosage of .1 mg dibenz[*a*,*h*]anthracene dispersed in .25 cc water was selected as one which would give a response in a range of number of nodules suitable for detecting any significant change in response brought about by exposure to high concentration of oxygen. Since it was known that the crystals of the carcinogen become lodged in the capillaries of the lung it appeared that the sequence of procedures most likely to give a positive result would be to inject the animals and immediately thereafter to expose them to the high concentration of oxygen in order that any effect of the oxygen would coincide with the action of the dibenz[*a*,*h*]anthracene.

For exposure of the animals to a high ambient concentration of oxygen, a flow line was constructed which contained a flow controller, a flowrate meter, and a gas-tight exposure chamber. Oxygen gas, supplied from high pressure cylinders through a two-stage pressure reducing valve, passed sequentially through the flow controller and the flowrate meter and then into the chamber. Copper tubing, ¼ inch in diameter, was used to complete the flow line except for a small segment of tygon tubing used to connect the flowrate meter and the chamber. The copper tubing connections were made gas-tight by the use of flare-nut unions. The flow controller and flowrate meter afforded a continuously regulated and monitored gas flow of 400 cc/min. ± 1 cc into the exposure chamber. The exposure chamber was a plexiglas cylinder, sealed at the base with a plexiglas plate and closed at the top with a removable lid. The lid was pressure sealed to the body of the cylinder by means of an O-ring gasket. The volume of the chamber was approximately 2500 cc and was large enough to allow simultaneous exposure of 4 animals. A pellet food hopper and a water bottle were contained within the chamber. The animals were maintained on a wire mesh floor suspended within the chamber. The oxygen gas used in these experiments was "medical therapy" grade. Mass spectrometric analysis revealed the average gas purity for all cylinders to be 99.4% ± .3%. The contaminating gas was nitrogen. The gas inlet orifice was located at the base of the chamber so that the flow path for the gas was upward over the animals to the gas outlet orifice located in the center of the chamber lid. This flow path afforded the best possible gas mixing within the chamber volume with effective removal of the respired atmosphere thus maintaining high, relatively constant ambient levels of oxygen concentration. Samples of the effluent gas from the chamber were obtained at the end of 24 hours of the 48 hour exposure period for mass spectrometric analysis.

Observations were made on a total of 60 exposed strain A mice consisting of 40 males and 20 females with a comparable number of nonexposed controls. All animals were approximately 2 months of age when injected and exposed. There were 15 experiments each of which included 4 experimental mice all of one sex and 4 of their littermate controls of the same sex. During the course of the experiments one control was eliminated from each of 4 experiments. Both the controls and the experimental animals were injected with the carcinogen in the lateral vein of the tail and approximately 30 minutes later the experimental animals were sealed in the exposure chamber while the controls remained in the air of the animal room. The exposure lasted for 48 hours for all experiments but one in which it was extended for 96 hours. After the exposure the experimental animals were returned to the air of the animal room. Following these procedures the animals were kept in plastic cages in the animal room with 8 animals to the cage and segregated as to sex and as to controls and exposed animals. They were given Derwood pelleted food and tap water *ad libitum*. All mice were

weighed at the time they were injected; the exposed mice were weighed at the time they were removed from the chamber; and all were weighed thereafter at weekly intervals. All animals were killed and examined for tumors 4 months after the injection and exposure. The lungs were injected intratracheally with approximately 1 cc of Fekete's modification of Tellyesniczky's fixative (70 percent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) before the chest cavity was opened. The lungs were then removed, the lobes were separated and the tumors appearing on the surface of each lobe were counted with the aid of the dissecting microscope. Later, at least one nodule from each animal was sectioned and stained with hematoxylin and eosin for histologic examination. The eyes were routinely sectioned and stained, but no histologic changes were observed. The animals were examined for other neoplasms but none was found.

Results. The animals in the exposure chamber could be visualized at all times and the exposure procedures appeared to be tolerated well. No deaths occurred in the experimental groups during or after the exposure. Analysis of effluent gas samples obtained from the chamber showed an average oxygen depletion of 1.0%, varying for individual groups from .83% to 1.13%. The average CO_2 content was .80% varying from .71% to .91%. The average R.Q. was .84 varying from .80 to .86. The exposed mice showed a slight average reduction in weight during the exposure period but thereafter they maintained weights comparable with those of the controls.

TABLE I. Occurrence of Pulmonary Tumors in Mice 4 Months after Injection with 0.1 mg Dibenz[*a,h*]anthracene with and without Subsequent 48 Hr Exposure to Approximately 100% Oxygen. 15 experiments.

Mice exposed to oxygen			Mice kept in air		
No.	Sex	Avg No. tumors	No.	Sex	Avg No. tumors
4	♂	28.25	4	♂	10.25
4	♂	24.00	4	♂	14.25
4	♀	20.75	3	♀	6.67
4	♂	29.50	3	♂	18.00
*4	♂	85.00	4	♂	63.75
4	♂	38.50	4	♂	28.75
†4	♂	44.00	4	♂	33.00
4	♀	32.75	4	♀	19.00
4	♀	31.25	4	♀	29.75
4	♂	48.00	4	♂	24.75
‡4	♂	31.25	4	♂	26.25
4	♂	11.50	4	♂	13.50
4	♂	22.00	4	♂	16.00
4	♀	36.50	3	♀	27.33
4	♀	37.50	3	♀	28.67

* Injected with 0.2 mg DBA.
† Autopsy 4.5 mo after injection and exposure.
‡ Exposed to oxygen for 96 hr.

The results presented in Table I clearly show that high oxygen administration to strain A mice for 48 hours immediately following injection of dibenz[*a,h*]anthracene resulted in an increase in number of pulmonary tumors over that of those that only received the carcinogen. Some difference in dosage of the carcinogen resulting in some variations between experiments is apparent as may be expected with a dispersion of this kind. It is obvious that there was some increase in concentration of the dispersion as the material was taken from the container in successive experiments. There were also factors causing individual variations but these can be considered randomly distributed within each experiment. In all experiments the exposed mice had on the average more tumors than did the controls with the exception of experiment 12 in which the average number of tumors in the controls was slightly higher than that observed in the experimental animals. Among the other experiments in which the exposed animals had more tumors, 2- and 3-fold differences were observed. The probability that this 14-1 distribution could have been a chance variation from a 50-50 distribution is .0005.

Histologically these tumors appeared as adenomas typical of those that have been observed to occur spontaneously or following injection of a carcinogen. Aside from the tumors no unusual changes were noted in the tissues of the lung.

Discussion. Further study will be necessary before offering any discussion on the mechanism by which the exposure to high concentration of oxygen results in an increase in occurrence of pulmonary tumors in mice. The parallel between the effect of high concentration of oxygen on mutation rate and its

effect on induction of pulmonary tumors is of interest, but it must be suspected that agents capable of producing chromosomal changes may also produce other changes of the cell and that some of these other changes may be associated with carcinogenesis.

Data on pulmonary tumors that can be related to the present study were published by Klein(7) who was studying transplacental passage of urethan in the induction of pulmonary tumors in mice. He noted a greater number of nodules in one series of mice that were born by caesarean section and were treated differently from those in the other series in a number of other ways including being placed for a short time under a funnel through which 95% oxygen flowed. He suggested the over-all handling of the caesarean born animals might have increased dosage of urethan, or affected fetal lung metabolism or the metabolism or elimination of the urethan, but he did not report any attempt to identify which of the variables was responsible. From the observations reported herein one could assume that the increase in number of nodules he observed was due in part at least to the exposure to the oxygen.

From the standpoint of future experimentation, the importance of the demonstration of an effect of molecular oxygen on the neoplastic process can not be minimized. Caution should be taken, however, in any attempt to relate directly these observations on pulmonary tumors in mice with the problem of pulmonary cancer in man. It is well recognized that the pulmonary tumor of the mouse is not comparable to the usual lung cancer in man either in respect to its cell of origin or its usual degree of malignancy. Furthermore, a potent carcinogen, dibenz[*a,h*]anthracene, was used in the study reported herein.

Summary. Strain A mice injected at 2 months of age with dibenz[*a,h*]anthracene and immediately thereafter exposed for 48 hours to approximately 100% oxygen had when killed and examined 4 months later a significantly greater average number of pulmonary tumors than did the controls injected with dibenz[*a,h*]anthracene and kept in air.

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Effect of Choline on Phosphatide Metabolism of Choline-Deficient and Cholesterol-Fed Rabbits.* (22508)

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It has been amply demonstrated that the administration of a single dose of choline stimulates liver phosphatide turnover in choline-deficient animals(1). These findings have been interpreted(1) as evidence that choline exerts its lipotropic effect through its action on liver phosphatides. In the present study we have investigated the effect of choline on phosphatide metabolism of choline-deficient and of cholesterol fatty livers of rabbits and have compared the action of a single dose of choline with the effects of prolonged

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TABLE I. Phosphatide Concentrations and Specific Activities 6 Hr after P^{32}.

		High fat-low protein diet			
		No treatment	Single dose intrav. choline	Daily oral choline	Purina chow
No. of animals		5	6	6	9
Liver	mg P/g*	1.25 ± .09	1.16 ± .12	1.17 ± .04	1.06 ± .10
	s.a.†	22.6 ± 2.05	38.7 ± 5.13	13.5 ± 1.85	12.4 ± 1.18
Plasma	mg P/g	.071± .009	.068± .007	.094± .016	.026± .002
	s.a.	10.4 ± 2.21	16.1 ± 1.39	4.7 ± 1.01	6.2 ± .90
Aorta	mg P/g	.19 ± .02	.18 ± .02	.15 ± .01	.19 ± .01
	s.a.	12.2 ± 3.14	12.2 ± 3.02	8.5 ± 1.12	7.6 ± .90

* mg phosphatide P per g fresh tissue or per ml plasma.
† Specific activity is % of injected P^{32}/g phosphatide P. Numbers following ± are stand. errors.

administration of this agent.

Methods. Albino rabbits weighing 1.5-3.1 kg were placed on Purina rabbit chow for at least 2 weeks and then were either maintained on this diet or transferred to one of the various experimental regimens. One group of 11 rabbits consumed approximately 30 g/day of a high-fat, low-protein, choline-deficient diet‡(2) for a period of 14 days. Another group of 6 rabbits were fed the same diet, to which was added 0.3 g of choline chloride§ per day. When the diet was fed in a frozen state the animals consumed it readily. Experimental atheromatosis was induced by supplementing Purina rabbit chow with 1% cholesterol§ and 2.8% vegetable fat‖ for 5 months. At the termination of the dietary period 0.5 mc of radioactive phosphate (P^{32}) was administered intravenously to experimental and control animals. Simultaneously, some of the animals were given one intravenous injection of 20 mg of choline chloride/kg of body wt as a 1% solution.¶ Six hours later, after the withdrawal of a terminal blood sample, all rabbits were killed with intracardiac Na-pentobarbital. Tissue lipids were extracted with alcohol and ethyl ether, evaporated under vacuum, and reextracted with petroleum ether. Aliquots of the petroleum ether extract were analyzed for radioactive and chemical phosphatide phosphorus(3).

Results. Table I lists the concentrations and specific activities of liver, plasma and aortic phosphatides 6 hrs after intravenous P^{32}. The liver phosphatide concentration and specific activity in the animals on the high-fat, low-protein diet which was supplemented daily with 1% choline did not differ from the corresponding values in the animals fed Purina chow. Thus it would appear that even though the high-fat, low-protein, choline-supplemented diet differed considerably from the Purina chow, a normal liver phosphatide metabolism was maintained on the synthetic diet. Removal of choline from this diet (no treatment column) resulted, however, in a 70% increase in the specific activity of the liver phosphatides. A further 70% elevation in specific activity was achieved by a single intravenous dose of choline. Thus, a single dose of intravenous or oral(1) choline appears to stimulate liver phosphatide synthesis whereas daily addition of choline to the high-fat diet seems to depress liver phosphatide synthesis below that observed in the untreated animals. The observation that chronic choline supplements, in amounts sufficient to exert lipotropic action, result in depressed liver phosphatide turnover may reflect the greatly decreased amount of fatty substrate in the livers of the supplemented animals as compared to that present in the choline-deficient group.

The plasma data follow the same pattern as those of liver, which is not surprising in

‡ Diet had the following composition: 38% lard, 44% sucrose, 8% vitamin test casein, 3% brewer's yeast, 2% cod liver oil and 5% Cowgill's salt mixture.

§ Cholesterol and choline chloride for feeding was donated by Merck and Co.

‖ The Humko Co., Memphis.

¶ Sterile choline chloride donated by the Fine Chemical Division, American Cyanamid Co.

TABLE II. Phosphatide Concentrations and Specific Activities of Cholesterol-Fed Rabbits 6 Hr after P^{32}.

		No treatment	Single dose intrav. choline
	No. of animals	4	4
Liver	mg P/g*	1.27 ± .24	1.27 ± .19
	s.a.†	17.9 ± 1.4	17.9 ± .8
Plasma	mg P/g	.233 ± .024	.195 ± .025
	s.a.	3.8 ± .61	3.7 ± .44

* mg phosphatide P per g fresh tissue or per ml of plasma.

† Specific activity is % of inj. P^{32}/g phosphatide P. Numbers following ± are stand. errors.

view of the fact that a considerable portion of the plasma phosphatides are derived from the liver(4). All animals on the high-fat diet showed a 3- to 4-fold increase in the concentration of plasma phosphatides and only small differences within the various experimental groups. This is in agreement with the observation of Bloor(5) that the administration of high-fat diets to rabbits resulted in a marked increase in plasma phosphatide, cholesterol and fatty acids. The aorta data are included in Table I because of our previous demonstration that on a high-cholesterol diet the phosphatide turnover in the thoracic aorta was markedly increased(6). The data (bottom row) indicate that the high-fat, low-protein regimen did not affect aortic phosphatide synthesis and that a single dose of choline, which had a marked stimulatory effect on liver phosphatide synthesis, did not alter the synthesis of these lipides in aorta. Apparently, the phosphatide turnover of aorta is regulated by factors other than those which influence this process in liver.

Since the cholesterol-fed rabbit is characterized by severe lipide accumulation in liver and plasma, it appeared of interest to study the effect of choline on phosphatide metabolism of this animal. The data in Table II refer to the effects of intravenous choline on the phosphatide metabolism of rabbits maintained for 5 months on a 1% cholesterol intake. Noteworthy are the markedly increased levels of plasma phosphatides to values even exceeding those of the animals on the high-fat diets. The specific activity data demonstrate that the injection of choline had no stimulatory effect on the synthesis of liver or plasma phosphatides, an indication that these animals were not choline-deficient.

Summary. In rabbits maintained on a high-fat, low-protein, choline-deficient diet for a period of 14 days, the synthesis of liver phosphatides was markedly increased. A single dose of intravenous choline was associated with a further increment in phosphatide synthesis, while chronic choline supplements depressed liver phosphatide synthesis. The plasma phosphatide concentration and synthesis were markedly elevated in all the fat-fed animals. The aortic phosphatide synthesis remained unaltered. Choline administration to cholesterol-fed rabbits did not alter the plasma or liver phosphatide metabolism.

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